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(54) Title: 56115, A NOVEL HUMAN TWIK POTASSIUM CHANNEL AND USES THEREFOR

(57) Abstract: The invention provides isolated nucleic acid molecules, designated TWIK-9 nucleic acid molecules, which encode novel TWIK-related potassium channel subunit molecules. The innovation also provides antisense nucleic acid molecules, recombinant expression vectors containing TWIK-9 nucleic acid molecules, host cells into which the expression vectors have been introduced, and nonhuman transgenic animals in which a TWIK-9 gene has been introduced or disrupted. The invention still further provides isolated TWIK-9 proteins, fusion proteins, antigenic peptides and anti-TWIK-9 antibodies. Diagnostic methods utilizing compositions of the invention are also provided.

## 56115, A NOVEL HUMAN TWIK POTASSIUM CHANNEL AND USES THEREFOR

### Related Applications

- 5           This application claims the benefit of U.S. Provisional Patent Application Serial No.: 60/235,059 filed on September 25, 2000, incorporated herein in its entirety by this reference.

### Background of the Invention

- 10           Potassium ( $K^+$ ) channels are ubiquitous proteins which are involved in the setting of the resting membrane potential as well as in the modulation of the electrical activity of cells. In excitable cells,  $K^+$  channels influence action potential waveforms, firing frequency, and neurotransmitter secretion (Rudy, B. (1988) *Neuroscience*, 25, 729-749; Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd Ed.). In non-  
15   excitable cells, they are involved in hormone secretion, cell volume regulation and potentially in cell proliferation and differentiation (Lewis *et al.* (1995) *Annu. Rev. Immunol.*, 13, 623-653). Developments in electrophysiology have allowed the identification and the characterization of an astonishing variety of  $K^+$  channels that differ in their biophysical properties, pharmacology, regulation and tissue distribution  
20   (Rudy, B. (1988) *Neuroscience*, 25, 729-749; Hille, B. (1992) *Ionic Channels of Excitable Membranes*, 2nd Ed.). More recently, cloning efforts have shed considerable light on the mechanisms that determine this functional diversity. Furthermore, analyses of structure-function relationships have provided an important set of data concerning the molecular basis of the biophysical properties (selectivity, gating, assembly) and the  
25   pharmacological properties of cloned  $K^+$  channels.

- Functional diversity of  $K^+$  channels arises mainly from the existence of a great number of genes coding for pore-forming subunits, as well as for other associated regulatory subunits. Two main structural families of pore-forming subunits have been identified. The first one consists of subunits with a conserved hydrophobic core  
30   containing six transmembrane domains (TMDs). These  $K^+$  channel  $\alpha$  subunits participate in the formation of outward rectifier voltage-gated ( $K_v$ ) and  $Ca^{2+}$ -dependent  $K^+$  channels. The fourth TMD contains repeated positive charges involved in the voltage gating of these channels and hence in their outward rectification (Logothetis *et al.* (1992) *Neuron*, 8, 531-540; Bezanilla *et al.* (1994) *Biophys. J.* 66, 1011-1021).

- 35           The second family of pore-forming subunits have only two TMDs. They are essential subunits of inward-rectifying (IRK), G-protein-coupled (GIRK) and ATP-sensitive ( $K_{ATP}$ )  $K^+$  channels. The inward rectification results from a voltage-dependent block by cytoplasmic  $Mg^{2+}$  and polyamines (Matsuda, H. (1991) *Annu. Rev.*

*Physiol.*, 53, 289-298). A conserved domain, called the P domain, is present in all members of both families (Pongs, O. (1993) *J. Membr. Biol.*, 136, 1-8; Heginbotham *et al.* (1994) *Biophys. J.* 66,1061-1067; Mackinnon, R. (1995) *Neuron*, 14, 889-892; Pascual *et al.*, (1995) *Neuron*, and 14, 1055-1063). This domain is an essential element  
5 of the aqueous K<sup>+</sup>-selective pore. In both groups, the assembly of four subunits is necessary to form a functional K<sup>+</sup> channel (Mackinnon, R. (1991) *Nature*, 350, 232-235; Yang *et al.*, (1995) *Neuron*, 15, 1441-1447).

In both six TMD and two TMD pore-forming subunit families, different subunits coded by different genes can associate to form heterotetramers with new channel  
10 properties (Isacoff *et al.*, (1990) *Nature*, 345, 530-534). A selective formation of heteropolymeric channels may allow each cell to develop the best K<sup>+</sup> current repertoire suited to its function. Pore-forming  $\alpha$  subunits of Kv channels are classified into different subfamilies according to their sequence similarity (Chandy *et al.* (1993) *Trends Pharmacol. Sci.*, 14: 434). Tetramerization is believed to occur preferentially between  
15 members of each subgroup (Covarrubias *et al.* (1991) *Neuron*, 7, 763-773). The domain responsible for this selective association is localized in the N-terminal region and is conserved between members of the same subgroup. This domain is necessary for hetero- but not homo-multimeric assembly within a subfamily and prevents co-assembly between subfamilies. Recently, pore-forming subunits with two TMDs were also shown  
20 to co-assemble to form heteropolymers (Duprat *et al.* (1995) *Biochem. Biophys. Res. Commun.*, 212, 657-663. This heteropolymerization seems necessary to give functional GIRKs. IRKs are active as homopolymers but also form heteropolymers.

New structural types of K<sup>+</sup> channels were identified recently in both humans and yeast. These channels have two P domains in their functional subunit instead of only  
25 one (Ketchum *et al.* (1995) *Nature*, 376, 690-695; Lesage *et al.* (1996) *J. Biol. Chem.*, 271, 4183-4187; Lesage *et al.* (1996) *EMBO J.*, 15, 1004-1011; Reid *et al.* (1996) *Receptors Channels* 4, 51-62). The human channel called TWIK-1, has four TMDs. TWIK-1 is expressed widely in human tissues and is particularly abundant in the heart and the brain. TWIK-1 currents are time independent and inwardly rectifying. These  
30 properties suggest that TWIK-1 channels are involved in the control of the background K<sup>+</sup> membrane conductance (Lesage *et al.* (1996) *EMBO J.*, 15, 1004-1011).

### Summary of the Invention

The present invention is based, at least in part, on the discovery of novel  
35 members of the TWIK (for Tandem of P domains in a Weak Inward rectifying K<sup>+</sup> channel)-like family of potassium channels, referred to herein as TWIK-9 nucleic acid and protein molecules. The TWIK-9 nucleic acid and protein molecules of the present invention are useful as targets for developing modulating agents to regulate a variety of

cellular processes. Accordingly, in one aspect, this invention provides isolated nucleic acid molecules encoding TWIK-9 proteins or biologically active portions thereof, as well as nucleic acid fragments suitable as primers or hybridization probes for the detection of TWIK-9-encoding nucleic acids.

5 In one embodiment, the invention features an isolated nucleic acid molecule that includes the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3. In another embodiment, the invention features an isolated nucleic acid molecule that encodes a polypeptide including the amino acid sequence set forth in SEQ ID NO:2. In another embodiment, the invention features an isolated nucleic acid molecule that includes the  
10 nucleotide sequence contained in the plasmid deposited with ATCC® as Accession Number \_\_\_\_\_.

In still other embodiments, the invention features isolated nucleic acid molecules including nucleotide sequences that are substantially identical (*e.g.*, 60% identical) to the nucleotide sequence set forth as SEQ ID NO:1 or SEQ ID NO:3. The invention further  
15 features isolated nucleic acid molecules including at least 30 contiguous nucleotides of the nucleotide sequence set forth as SEQ ID NO:1 or SEQ ID NO:3. In another embodiment, the invention features isolated nucleic acid molecules which encode a polypeptide including an amino acid sequence that is substantially identical (*e.g.*, 60% identical) to the amino acid sequence set forth as SEQ ID NO:2. Also featured are  
20 nucleic acid molecules which encode allelic variants of the polypeptide having the amino acid sequence set forth as SEQ ID NO:2. In addition to isolated nucleic acid molecules encoding full-length polypeptides, the present invention also features nucleic acid molecules which encode fragments, for example, biologically active or antigenic fragments, of the full-length polypeptides of the present invention (*e.g.*, fragments  
25 including at least 10 contiguous amino acid residues of the amino acid sequence of SEQ ID NO:2). In still other embodiments, the invention features nucleic acid molecules that are complementary to, antisense to, or hybridize under stringent conditions to the isolated nucleic acid molecules described herein.

In a related aspect, the invention provides vectors including the isolated nucleic acid molecules described herein (*e.g.*, TWIK-9-encoding nucleic acid molecules). Such  
30 vectors can optionally include nucleotide sequences encoding heterologous polypeptides. Also featured are host cells including such vectors (*e.g.*, host cells including vectors suitable for producing TWIK-9 nucleic acid molecules and polypeptides).

35 In another aspect, the invention features isolated TWIK-9 polypeptides and/or biologically active or antigenic fragments thereof. Exemplary embodiments feature a polypeptide including the amino acid sequence set forth as SEQ ID NO:2, a polypeptide including an amino acid sequence at least 60% identical to the amino acid sequence set

forth as SEQ ID NO:2, a polypeptide encoded by a nucleic acid molecule including a nucleotide sequence at least 60% identical to the nucleotide sequence set forth as SEQ ID NO:1 or SEQ ID NO:3. Also featured are fragments of the full-length polypeptides described herein (*e.g.*, fragments including at least 10 contiguous amino acid residues of the sequence set forth as SEQ ID NO:2) as well as allelic variants of the polypeptide having the amino acid sequence set forth as SEQ ID NO:2.

The TWIK-9 polypeptides and/or biologically active or antigenic fragments thereof, are useful, for example, as reagents or targets in assays applicable to treatment and/or diagnosis of TWIK-9 mediated or potassium channel related disorders. In one embodiment, a TWIK-9 polypeptide or fragment thereof has a TWIK-9 activity. In another embodiment, a TWIK-9 polypeptide or fragment thereof has at least one or more of the following domains: at least one, preferably two, three or four transmembrane domains, at least one, preferably two pore loops (P-loops), and optionally, has a TWIK-9 activity. In a related aspect, the invention features antibodies (*e.g.*, antibodies which specifically bind to any one of the polypeptides, as described herein) as well as fusion polypeptides including all or a fragment of a polypeptide described herein.

The present invention further features methods for detecting TWIK-9 polypeptides and/or TWIK-9 nucleic acid molecules, such methods featuring, for example, a probe, primer or antibody described herein. Also featured are kits for the detection of TWIK-9 polypeptides and/or TWIK-9 nucleic acid molecules. In a related aspect, the invention features methods for identifying compounds which bind to and/or modulate the activity of a TWIK-9 polypeptide or TWIK-9 nucleic acid molecule described herein. Also featured are methods for modulating a TWIK-9 activity.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

### **Brief Description of the Drawings**

*Figure 1* depicts the nucleotide sequence of the human TWIK-9 cDNA and the corresponding amino acid sequence. The nucleotide sequence corresponds to nucleic acids 1 to 1262 of SEQ ID NO:1. The amino acid sequence corresponds to amino acids 1-374 of SEQ ID NO:2. The coding region without the 5' or 3' untranslated regions of the human TWIK-9 gene is shown in SEQ ID NO:3.

*Figure 2* depicts a structural, hydrophobicity, and antigenicity analysis of the human TWIK-9 protein.

*Figure 3* depicts the results of a search which was performed against the Washington University HMM database and which resulted in the identification of the protein of SEQ ID NO:2 as a TWIK channel family member.

**Detailed Description of the Invention**

The present invention is based, at least in part, on the discovery of novel molecules, referred to herein as TWIK-9 nucleic acid and protein molecules, which are novel members of the TWIK (for Tandem of P domains in a Weak Inward rectifying K<sup>+</sup> channel)-like family of potassium channels. These novel molecules are capable of, for example, modulating a potassium channel mediated activity in a cell, *e.g.*, a neuronal cell.

As used herein, a "potassium channel" includes a protein or polypeptide which is involved in receiving, conducting, and transmitting signals in an electrically excitable cell, *e.g.*, a neuronal cell, or a muscle cell (*e.g.*, a cardiac muscle cell). Potassium channels are potassium ion selective, and can determine membrane excitability (the ability of, for example, a neuron to respond to a stimulus and convert it into an impulse). Potassium channels can also influence the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation. Potassium channels are typically expressed in electrically excitable cells, *e.g.*, neurons, muscle, endocrine, and egg cells, and may form heteromultimeric structures, *e.g.*, composed of pore-forming  $\alpha$  and cytoplasmic  $\beta$  subunits. Potassium channels may also be found in non-excitabile cells (*e.g.*, spleen cells or prostate cells), where they may play a role in, *e.g.*, signal transduction. Examples of potassium channels include: (1) the voltage-gated potassium channels, (2) the ligand-gated potassium channels, *e.g.*, neurotransmitter-gated potassium channels, and (3) cyclic-nucleotide-gated potassium channels. Voltage-gated and ligand-gated potassium channels are expressed in the brain, *e.g.*, in brainstem monoaminergic and forebrain cholinergic neurons, where they are involved in the release of neurotransmitters, or in the dendrites of hippocampal and neocortical pyramidal cells, where they are involved in the processes of learning and memory formation. For a detailed description of potassium channels, see Kandel E.R., *et al.*, Principles of Neural Science, second edition, (Elsevier Science Publishing Co., Inc., N.Y. (1985)), the contents of which are incorporated herein by reference. As the TWIK-like proteins of the present invention may modulate potassium channel mediated activities, they may be useful for developing novel diagnostic and therapeutic agents for potassium channel associated disorders.

As used herein, a "potassium channel mediated activity" includes an activity which involves a potassium channel, *e.g.*, a potassium channel in a neuronal cell, or a muscle cell (*e.g.*, a cardiac muscle cell), associated with receiving, conducting, and transmitting signals in, for example, the nervous system. Potassium channel mediated activities include release of neurotransmitters, *e.g.*, dopamine or norepinephrine, from cells, *e.g.*, neuronal cells; modulation of resting potential of membranes, wave forms and

frequencies of action potentials, and thresholds of excitation; and modulation of processes such as integration of sub-threshold synaptic responses, the conductance of back-propagating action potentials in, for example, neuronal cells or muscle cells, participation in signal transduction pathways, and participation in nociception.

5 As used herein, a "potassium channel associated disorder" includes a disorder, disease or condition which is characterized by a misregulation of a potassium channel mediated activity. Potassium channel associated disorders can detrimentally affect conveyance of sensory impulses from the periphery to the brain and/or conductance of motor impulses from the brain to the periphery; integration of reflexes; interpretation of  
10 sensory impulses; and emotional, intellectual (*e.g.*, learning and memory), or motor processes.

Examples of potassium channel associated disorders include central nervous system (CNS) disorders such as cognitive and neurodegenerative disorders, examples of which include, but are not limited to, Alzheimer's disease, dementias related to  
15 Alzheimer's disease (such as Pick's disease), Parkinson's and other Lewy diffuse body diseases, senile dementia, Huntington's disease, Gilles de la Tourette's syndrome, multiple sclerosis, amyotrophic lateral sclerosis, movement disorders, progressive supranuclear palsy, epilepsy, AIDS related dementia, and Jakob-Creutzfeldt disease; autonomic function disorders such as hypertension and sleep disorders, and  
20 neuropsychiatric disorders, such as depression, schizophrenia, schizoaffective disorder, korsakoff's psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder, psychoactive substance use disorders, anxiety, phobias, panic disorder, as well as bipolar  
25 affective disorder, *e.g.*, severe bipolar affective (mood) disorder (BP-1), and bipolar affective neurological disorders, *e.g.*, migraine and obesity. Further CNS-related disorders include, for example, those listed in the American Psychiatric Association's Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version of which is incorporated herein by reference in its entirety.

30 Further examples of potassium channel associated disorders include cardiac-related disorders. Cardiovascular system disorders in which the TWIK-9 molecules of the invention may be directly or indirectly involved include arteriosclerosis, ischemia reperfusion injury, restenosis, arterial inflammation, vascular wall remodeling, ventricular remodeling, rapid ventricular pacing, coronary microembolism, tachycardia,  
35 bradycardia, pressure overload, aortic bending, coronary artery ligation, vascular heart disease, atrial fibrillation, Jervell syndrome, Lange-Nielsen syndrome, long-QT syndrome, congestive heart failure, sinus node dysfunction, angina, heart failure, hypertension, atrial fibrillation, atrial flutter, dilated cardiomyopathy, idiopathic

cardiomyopathy, myocardial infarction, coronary artery disease, cerebral ischemia, stroke, coronary artery spasm, and arrhythmia. TWIK-9-mediated or related disorders also include disorders of the musculoskeletal system such as paralysis and muscle weakness, *e.g.*, ataxia, myotonia, and myokymia.

- 5 Further disorders in which the TWIK-9 molecules of the invention may be involved are pain disorders. Pain disorders include those that affect pain signaling mechanisms. As used herein, the term "pain signaling mechanisms" includes the cellular mechanisms involved in the development and regulation of pain, *e.g.*, pain elicited by noxious chemical, mechanical, or thermal stimuli, in a subject, *e.g.*, a mammal such as a
- 10 human. In mammals, the initial detection of noxious chemical, mechanical, or thermal stimuli, a process referred to as "nociception", occurs predominantly at the peripheral terminals of specialized, small diameter sensory neurons. These sensory neurons transmit the information to the central nervous system, evoking a perception of pain or discomfort and initiating appropriate protective reflexes. The TWIK-9 molecules of the
- 15 present invention may be present on these sensory neurons and, thus, may be involved in detecting these noxious chemical, mechanical, or thermal stimuli and transducing this information into membrane depolarization events. Thus, the TWIK-9 molecules by participating in pain signaling mechanisms, may modulate pain elicitation and act as targets for developing novel diagnostic targets and therapeutic agents to control pain.
- 20 Examples of pain disorders include headache (*e.g.*, tension headache or migraine), back pain, cancer pain, arthritis pain, or neurogenic pain.

- Potassium channel disorders also include cellular proliferation, growth, differentiation, or migration disorders. Cellular proliferation, growth, differentiation, or migration disorders include those disorders that affect cell proliferation, growth,
- 25 differentiation, or migration processes. As used herein, a "cellular proliferation, growth, differentiation, or migration process" is a process by which a cell increases in number, size or content, by which a cell develops a specialized set of characteristics which differ from that of other cells, or by which a cell moves closer to or further from a particular location or stimulus. The TWIK-9 molecules of the present invention are involved in
- 30 signal transduction mechanisms, which are known to be involved in cellular growth, differentiation, and migration processes. Thus, the TWIK-9 molecules may modulate cellular growth, differentiation, or migration, and may play a role in disorders characterized by aberrantly regulated growth, differentiation, or migration. Such disorders include cancer, *e.g.*, carcinoma, sarcoma, lymphoma or leukemia, examples of
- 35 which include, but are not limited to, breast, endometrial, ovarian, uterine, hepatic, gastrointestinal, prostate, colorectal, and lung cancer, melanoma, neurofibromatosis, adenomatous polyposis of the colon, Wilms' tumor, nephroblastoma, teratoma, rhabdomyosarcoma; tumor invasion, angiogenesis and metastasis; skeletal dysplasia;



neuronal deficiencies resulting from impaired neural induction and patterning; and hematopoietic and/or myeloproliferative disorders.

TWIK-9-associated or related disorders also include disorders of tissues in which TWIK-9 protein is predominantly expressed, *e.g.*, dorsal root ganglia, cerebellum, testes,  
5 mammary gland, fetal brain and fetal thymus.

The term "family" when referring to the protein and nucleic acid molecules of the invention is intended to mean two or more proteins or nucleic acid molecules having a common structural domain or motif and having sufficient amino acid or nucleotide sequence homology as defined herein. Such family members can be naturally or non-  
10 naturally occurring and can be from either the same or different species. For example, a family can contain a first protein of human origin as well as other distinct proteins of human origin or alternatively, can contain homologues of non-human origin, *e.g.*, rat or mouse proteins. Members of a family can also have common functional characteristics.

For example, the family of TWIK-9 proteins comprises at least one  
15 "transmembrane domain" and preferably two, three, or four transmembrane domains. As used herein, the term "transmembrane domain" includes an amino acid sequence of about 15 amino acid residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes about at least 20, 25, 30, 35, 40, or 45 amino acid residues and spans the plasma membrane. Transmembrane domains are rich  
20 in hydrophobic residues, and typically have an alpha-helical structure. In a preferred embodiment, at least 50%, 60%, 70%, 80%, 90%, 95% or more of the amino acids of a transmembrane domain are hydrophobic, *e.g.*, leucines, isoleucines, tyrosines, or tryptophans. Transmembrane domains are described in, for example, Zagotta W.N. *et al.* (1996) *Annual Rev. Neurosci.* 19: 235-263, the contents of which are incorporated  
25 herein by reference. Amino acid residues 9-26, 108-125, 159-178 and 219-243 of the TWIK-9 protein are predicted to comprise transmembrane domains.

In another embodiment, a TWIK-9 molecule of the present invention is identified based on the presence of at least one, preferably two pore loops (P-loops). As used herein, the term "pore loop" or "P-loop" includes an amino acid sequence of about 15-45  
30 amino acid residues in length, preferably about 15-35 amino acid residues in length, and most preferably about 15-25 amino acid residues in length, which is involved in lining the potassium channel pore. A P-loop is typically found between transmembrane domains of potassium channels and is believed to be a major determinant of ion selectivity in potassium channels. Preferably, P-loops contain a G-[HYDROPHOBIC  
35 AMINO ACID]-G sequence, *e.g.*, a GYG, GLG, or GFG sequence. P-loops are described in, for example, Warmke *et al.* (1991) *Science* 252:1560-1562; Zagotta W.N. *et al.*, (1996) *Annual Rev. Neurosci.* 19:235-63 (Pongs, O. (1993) *J. Membr. Biol.*, 136, 1-8; Heginbotham *et al.* (1994) *Biophys. J.* 66,1061-1067; Mackinnon, R. (1995)

*Neuron*, and 14, 889-892; Pascual et al., (1995) *Neuron*, 14, 1055-1063), the contents of which are incorporated herein by reference. Amino acid residues 80-97 and 190-208 of the human TWIK-9 protein comprise a P-loop.

In another embodiment, a TWIK-9 molecule of the present invention is identified  
5 based on a high scoring match with a TWIK channel Hidden Markov Model (*i.e.*, a score of greater than 100, greater than 200, greater than 300, greater than 400 or greater than 500). To identify a high scoring match between a protein of interest and a TWIK channel HMM, the amino acid sequence of the protein is searched against a database of known HMMs (*e.g.*, the Washington University HMM database). The TWIK channel  
10 (HMM) has been assigned the PFAM Accession PF02034 (<http://genome.wustl.edu/Pfam/html>). A search was performed against the HMM database resulting in a high scoring match between SEQ ID NO:2 and the TWIK channel HMM, *e.g.*, a match having a score of greater than 587. The results of the search are set forth in Figure 3.

15 In a preferred embodiment, the TWIK-9 molecules of the invention include at least one and, preferably, two, three, or four transmembrane domains, and at least one, preferably two P loops. In another preferred embodiment, the TWIK-9 molecules of the invention include at least one and, preferably, two, three, or four transmembrane domains, and at least one, preferably two P loops, and have a high scoring match with a  
20 TWIK channel HMM.

A description of the Pfam database can be found in Sonhammer *et al.* (1997) *Proteins* 28:405-420, and a detailed description of HMMs can be found, for example, in Gribskov *et al.* (1990) *Meth. Enzymol.* 183:146-159; Gribskov *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:4355-4358; Krogh *et al.* (1994) *J. Mol. Biol.* 235:1501-1531; and  
25 Stultz *et al.* (1993) *Protein Sci.* 2:305-314, the contents of which are incorporated herein by reference.

The signature patterns or consensus patterns described herein are described according to the following designation: all amino acids are indicated according to their universal single letter designation; "x" designates any amino acid; x(n) designates n  
30 number of amino acids, *e.g.*, x (2) designates any two amino acids, *e.g.*, x (1-3) designates any of one to three amino acids; and, amino acids in brackets indicates any one of the amino acids within the brackets, *e.g.*, [RK] indicates any of one of either R (arginine) or K (lysine).

Isolated proteins of the present invention, preferably TWIK-9 proteins, have an amino acid sequence sufficiently homologous to the amino acid sequence of SEQ ID NO:2, or are encoded by a nucleotide sequence sufficiently homologous to SEQ ID NO:1 or 3. As used herein, the term “sufficiently homologous” refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (*e.g.*, an amino acid residue which has a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences share common structural domains or motifs and/or a common functional activity. For example, amino acid or nucleotide sequences which share common structural domains having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homology or identity across the amino acid sequences of the domains and contain at least one and preferably two structural domains or motifs, are defined herein as sufficiently homologous. Furthermore, amino acid or nucleotide sequences which share at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homology or identity and share a common functional activity are defined herein as sufficiently homologous.

In a preferred embodiment, a TWIK-9 protein includes at least one, preferably two, three, or four transmembrane domains, and at least one, preferably two pore loops (P-loops), and has an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous or identical to the amino acid sequence of SEQ ID NO:2, or the amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_. In yet another preferred embodiment, a TWIK-9 protein includes at least one, preferably two, three, or four transmembrane domains, and at least one, preferably two pore loops (P-loops), and is encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a complement of a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3. In another preferred embodiment, a TWIK-9 protein includes at least one, preferably two, three, or four transmembrane domains, and at least one, preferably two pore loops (P-loops), and has a TWIK-9 activity.

As used interchangeably herein, a “TWIK-9 activity”, “biological activity of TWIK-9” or “functional activity of TWIK-9”, includes an activity exerted or mediated by a TWIK-9 protein, polypeptide or nucleic acid molecule on a TWIK-9 responsive cell or tissue, or on a TWIK-9 substrate, as determined *in vivo* or *in vitro*, according to standard techniques. In one embodiment, a TWIK-9 activity is a direct activity, such as an association with a TWIK-9 target molecule. As used herein, a “target molecule” or “binding partner” is a molecule with which a TWIK-9 protein binds or interacts in

nature, such that TWIK-9-mediated function is achieved. A TWIK-9 target molecule can be a non-TWIK-9 molecule or a TWIK-9 protein or polypeptide of the present invention. In an exemplary embodiment, a TWIK-9 target molecule is a TWIK-9 ligand, *e.g.*, a potassium channel pore forming subunit or a potassium channel ligand. A  
5 TWIK-9 activity can also be an indirect activity, such as a cellular signaling activity mediated by interaction of the TWIK-9 protein with a TWIK-9 ligand or substrate. The biological activities of TWIK-9 are described herein.

For example, the TWIK-9 proteins of the present invention can have one or more of the following activities: (1) interacting with a non-TWIK protein molecule; (2)  
10 activating a TWIK-dependent signal transduction pathway; (3) modulating the release of neurotransmitters; (4) modulating membrane excitability; (5) influencing the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation, (6) modulating processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating  
15 action potentials, and (7) mediating nociception.

The nucleotide sequence of the isolated human TWIK-9 cDNA and the predicted amino acid sequence encoded by the TWIK-9 cDNA are shown in Figure 1 and in SEQ ID NO:1 and 2, respectively. A plasmid containing the human TWIK-9 cDNA was deposited with the American Type Culture Collection (ATCC), 10801 University  
20 Boulevard, Manassas, VA 20110-2209, on \_\_\_\_\_ and assigned Accession Number \_\_\_\_\_. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. This deposit were made merely as a convenience for those of skill in the art and is not an admission that a deposit is required under 35 U.S.C. §112.

25 The human TWIK-9 gene, which is approximately 1262 nucleotides in length, encodes a protein having a molecular weight of approximately 41 kD and which is approximately 374 amino acid residues in length.

Various aspects of the invention are described in further detail in the following subsections:

30

#### I. Isolated Nucleic Acid Molecules

One aspect of the invention pertains to isolated nucleic acid molecules that encode TWIK-9 proteins or biologically active portions thereof, as well as nucleic acid fragments sufficient for use as hybridization probes to identify TWIK-9-encoding  
35 nucleic acid molecules (*e.g.*, TWIK-9 mRNA) and fragments for use as PCR primers for the amplification or mutation of TWIK-9 nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA

generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regards to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated TWIK-9 nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, or a portion thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of the nucleic acid sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, as hybridization probes, TWIK-9 nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2<sup>nd</sup>, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_ can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_.

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

Furthermore, oligonucleotides corresponding to TWIK-9 nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

In one embodiment, an isolated nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1 or 3. This cDNA may comprise  
5 sequences encoding the human TWIK-9 protein (*e.g.*, the "coding region", from nucleotides 15-1139), as well as 5' untranslated sequence (nucleotides 1-14) and 3' untranslated sequences (nucleotides 1140-1262) of SEQ ID NO:1. Alternatively, the nucleic acid molecule can comprise only the coding region of SEQ ID NO:1 (*e.g.*, nucleotides 15-1139, corresponding to SEQ ID NO:3). Accordingly, in another  
10 embodiment, an isolated nucleic acid molecule of the invention comprises SEQ ID NO:3 and nucleotides 1-14 of SEQ ID NO:1. In yet another embodiment, the isolated nucleic acid molecule comprises SEQ ID NO:3 and nucleotides 1140-1262 of SEQ ID NO:1. In yet another embodiment, the nucleic acid molecule consists of the nucleotide sequence set forth as SEQ ID NO:1 or SEQ ID NO:3.

15 In still another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, or a portion of any of these nucleotide sequences. A nucleic acid molecule which is complementary to the  
20 nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, is one which is sufficiently complementary to the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, such that it can hybridize to the nucleotide sequence  
25 shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, thereby forming a stable duplex.

In still another embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50%, 55%, 60%,  
30 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to the nucleotide sequence shown in SEQ ID NO:1 or 3 (*e.g.*, to the entire length of the nucleotide sequence), or to the nucleotide sequence (*e.g.*, the entire length of the nucleotide sequence) of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, or a portion or complement of any of these nucleotide  
35 sequences. In one embodiment, a nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least (or no greater than) 50-100, 100-200, 200-300, 305, 305-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100, 1000-1200 or more nucleotides in length and hybridizes under stringent

hybridization conditions to a complement of a nucleic acid molecule of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_.

Moreover, the nucleic acid molecule of the invention can comprise only a portion  
5 of the nucleic acid sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, for example, a fragment which can be used as a probe or primer or a fragment encoding a portion of a TWIK-9 protein, *e.g.*, a biologically active portion of a TWIK-9 protein. The nucleotide sequence determined from the cloning of the TWIK-9 gene allows for  
10 the generation of probes and primers designed for use in identifying and/or cloning other TWIK-9 family members, as well as TWIK-9 homologues from other species. The probe/primer (*e.g.*, oligonucleotide) typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12 or 15, preferably  
15 about 20 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 consecutive nucleotides of a sense sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, of an anti-sense sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, or of a naturally  
20 occurring allelic variant or mutant of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_.

Exemplary probes or primers are at least (or no greater than) 12 or 15, 20 or 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 or more nucleotides in length and/or comprise consecutive nucleotides of an isolated nucleic acid molecule described herein. Also  
25 included within the scope of the present invention are probes or primers comprising contiguous or consecutive nucleotides of an isolated nucleic acid molecule described herein, but for the difference of 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 bases within the probe or primer sequence. Probes based on the TWIK-9 nucleotide sequences can be used to detect (*e.g.*, specifically detect) transcripts or genomic sequences encoding the same or  
30 homologous proteins. In preferred embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. In another embodiment a set of primers is provided, *e.g.*, primers suitable for use in a PCR, which can be used to amplify a selected region of a TWIK-9 sequence, *e.g.*, a domain, region, site or other sequence  
35 described herein. The primers should be at least 5, 10, or 50 base pairs in length and less than 100, or less than 200, base pairs in length. The primers should be identical, or differ by no greater than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 bases when compared to a sequence disclosed herein or to the sequence of a naturally occurring variant. Such probes can be

used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a TWIK-9 protein, such as by measuring a level of a TWIK-9-encoding nucleic acid in a sample of cells from a subject, *e.g.*, detecting TWIK-9 mRNA levels or determining whether a genomic TWIK-9 gene has been mutated or deleted.

- 5           A nucleic acid fragment encoding a “biologically active portion of a TWIK-9 protein” can be prepared by isolating a portion of the nucleotide sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, which encodes a polypeptide having a TWIK-9 biological activity (the biological activities of the TWIK-9 proteins are described  
10   herein), expressing the encoded portion of the TWIK-9 protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the TWIK-9 protein. In an exemplary embodiment, the nucleic acid molecule is at least 50-100, 100-200, 200-300, 305, 305-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100, 1000-1200 or more nucleotides in length and encodes a protein having a  
15   TWIK-9 activity (as described herein).

- The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, due to degeneracy of the genetic code and thus encode the same TWIK-9 proteins as those  
20   encoded by the nucleotide sequence shown in SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_. In another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence which differs by at least 1, but no greater than 5, 10, 20, 50 or 100 amino acid residues from the amino  
25   acid sequence shown in SEQ ID NO:2, or the amino acid sequence encoded by the DNA insert of the plasmid deposited with the ATCC as Accession Number \_\_\_\_\_. In yet another embodiment, the nucleic acid molecule encodes the amino acid sequence of human TWIK-9. If an alignment is needed for this comparison, the sequences should be aligned for maximum homology.

- 30           Nucleic acid variants can be naturally occurring, such as allelic variants (same locus), homologues (different locus), and orthologues (different organism) or can be non naturally occurring. Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can  
35   occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product).



Allelic variants result, for example, from DNA sequence polymorphisms within a population (*e.g.*, the human population) that lead to changes in the amino acid sequences of the TWIK-9 proteins. Such genetic polymorphism in the TWIK-9 genes may exist among individuals within a population due to natural allelic variation. As  
5 used herein, the terms “gene” and “recombinant gene” refer to nucleic acid molecules which include an open reading frame encoding a TWIK-9 protein, preferably a mammalian TWIK-9 protein, and can further include non-coding regulatory sequences, and introns.

Accordingly, in one embodiment, the invention features isolated nucleic acid  
10 molecules which encode a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, wherein the nucleic acid molecule hybridizes to a complement of a nucleic acid molecule comprising SEQ ID NO:1 or 3, for example, under stringent hybridization  
15 conditions.

Allelic variants of TWIK-9, *e.g.*, human TWIK-9, include both functional and non-functional TWIK-9 proteins. Functional allelic variants are naturally occurring amino acid sequence variants of the TWIK-9 protein that maintain the ability, for example, to bind a TWIK-9 ligand or substrate, and/or modulate pain signaling  
20 mechanisms, membrane excitability or neurotransmitter release. Functional allelic variants will typically contain only conservative substitution of one or more amino acids of SEQ ID NO:2, or substitution, deletion or insertion of non-critical residues in non-critical regions of the protein.

Non-functional allelic variants are naturally occurring amino acid sequence  
25 variants of the TWIK-9 protein, *e.g.*, human TWIK-9, that do not have the ability to either bind or interact with a TWIK-9 ligand or substrate, and/or modulate any of the TWIK-9 activities described herein. Non-functional allelic variants will typically contain a non-conservative substitution, a deletion, or insertion, or premature truncation of the amino acid sequence of SEQ ID NO:2, or a substitution, insertion, or deletion in  
30 critical residues or critical regions of the protein.

The present invention further provides non-human orthologues (*e.g.*, non-human orthologues of the human TWIK-9 protein). Orthologues of the human TWIK-9 protein are proteins that are isolated from non-human organisms and possess, for example, the same TWIK-9 ligand or substrate binding, and/or modulation of pain signaling  
35 mechanisms, membrane excitability or neurotransmitter release activities of the human TWIK-9 protein. Orthologues of the human TWIK-9 protein can readily be identified as comprising an amino acid sequence that is substantially homologous to SEQ ID NO:2.

Moreover, nucleic acid molecules encoding other TWIK-9 family members and, thus, which have a nucleotide sequence which differs from the TWIK-9 sequences of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_ are intended to be within the scope of the invention. For example, another TWIK-9 cDNA can be identified based on the nucleotide sequence of human TWIK-9. Moreover, nucleic acid molecules encoding TWIK-9 proteins from different species, and which, thus, have a nucleotide sequence which differs from the TWIK-9 sequences of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_ are intended to be within the scope of the invention. For example, a mouse or monkey TWIK-9 cDNA can be identified based on the nucleotide sequence of a human TWIK-9.

Nucleic acid molecules corresponding to natural allelic variants and homologues of the TWIK-9 cDNAs of the invention can be isolated based on their homology to the TWIK-9 nucleic acids disclosed herein using the cDNAs disclosed herein, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. Nucleic acid molecules corresponding to natural allelic variants and homologues of the TWIK-9 cDNAs of the invention can further be isolated by mapping to the same chromosome or locus as the TWIK-9 gene.

Orthologues, homologues and allelic variants can be identified using methods known in the art (*e.g.*, by hybridization to an isolated nucleic acid molecule of the present invention, for example, under stringent hybridization conditions). In one embodiment, an isolated nucleic acid molecule of the invention is at least 15, 20, 25, 30 or more nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_. In other embodiment, the nucleic acid is at least 50-100, 100-200, 200-300, 305, 305-400, 400-500, 500-600, 600-700, 700-800, 800-900, 900-1000, 1000-1100, 1000-1200 or more nucleotides in length.

As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences that are significantly identical or homologous to each other remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more preferably at least about 80%, even more preferably at least about 85% or 90% identical to each other remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, Inc. (1995), sections 2, 4, and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*,

Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9, and 11. A preferred, non-limiting example of stringent hybridization conditions includes hybridization in 4X sodium chloride/sodium citrate (SSC), at about 65-70°C (or alternatively hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed  
5 by one or more washes in 1X SSC, at about 65-70°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or alternatively hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency hybridization conditions includes hybridization  
10 in 4X SSC, at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited values, *e.g.*, at 65-70°C or at 42-50°C are also intended to be encompassed by the present invention. SSPE (1xSSPE is 0.15M NaCl, 10mM NaH<sub>2</sub>PO<sub>4</sub>, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1X  
15 SSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes each after hybridization is complete. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature ( $T_m$ ) of the hybrid, where  $T_m$  is determined according to the following equations. For hybrids less than 18 base pairs in  
20 length,  $T_m(^{\circ}\text{C}) = 2(\# \text{ of A} + \text{T bases}) + 4(\# \text{ of G} + \text{C bases})$ . For hybrids between 18 and 49 base pairs in length,  $T_m(^{\circ}\text{C}) = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G} + \text{C}) - (600/\text{N})$ , where N is the number of bases in the hybrid, and  $[\text{Na}^+]$  is the concentration of sodium ions in the hybridization buffer ( $[\text{Na}^+]$  for 1X SSC = 0.165 M). It will also be recognized by the skilled practitioner that additional reagents may be added to  
25 hybridization and/or wash buffers to decrease non-specific hybridization of nucleic acid molecules to membranes, for example, nitrocellulose or nylon membranes, including but not limited to blocking agents (*e.g.*, BSA or salmon or herring sperm carrier DNA), detergents (*e.g.*, SDS), chelating agents (*e.g.*, EDTA), Ficoll, PVP and the like. When using nylon membranes, in particular, an additional preferred, non-limiting example of  
30 stringent hybridization conditions is hybridization in 0.25-0.5M NaH<sub>2</sub>PO<sub>4</sub>, 7% SDS at about 65°C, followed by one or more washes at 0.02M NaH<sub>2</sub>PO<sub>4</sub>, 1% SDS at 65°C (see *e.g.*, Church and Gilbert (1984) *Proc. Natl. Acad. Sci. USA* 81:1991-1995), or alternatively 0.2X SSC, 1% SDS.

Preferably, an isolated nucleic acid molecule of the invention that hybridizes  
35 under stringent conditions to the sequence of SEQ ID NO:1 or 3 corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

In addition to naturally-occurring allelic variants of the TWIK-9 sequences that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nucleotide sequences of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as

- 5 Accession Number \_\_\_\_\_, thereby leading to changes in the amino acid sequence of the encoded TWIK-9 proteins, without altering the functional ability of the TWIK-9 proteins. For example, nucleotide substitutions leading to amino acid substitutions at “non-essential” amino acid residues can be made in the sequence of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as
- 10 Accession Number \_\_\_\_\_. A “non-essential” amino acid residue is a residue that can be altered from the wild-type sequence of TWIK-9 (*e.g.*, the sequence of SEQ ID NO:2) without altering the biological activity, whereas an “essential” amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the TWIK-9 proteins of the present invention, *e.g.*, those present in a
- 15 transmembrane domain or P loop, are predicted to be particularly unamenable to alteration. Furthermore, additional amino acid residues that are conserved between the TWIK-9 proteins of the present invention and other members of the TWIK family are not likely to be amenable to alteration.

- Accordingly, another aspect of the invention pertains to nucleic acid molecules
- 20 encoding TWIK-9 proteins that contain changes in amino acid residues that are not essential for activity. Such TWIK-9 proteins differ in amino acid sequence from SEQ ID NO:2, yet retain biological activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 50%, 55%, 60%, 65%, 70%, 75%,
- 25 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% 99% or more homologous to SEQ ID NO:2, *e.g.*, to the entire length of SEQ ID NO:2.

- An isolated nucleic acid molecule encoding a TWIK-9 protein homologous to the protein of SEQ ID NO:2 can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO:1 or 3,
- 30 or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_ by standard techniques, such as site-
- 35 directed mutagenesis and PCR-mediated mutagenesis.

Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a

similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, 5 tyrosine, cysteine, tryptophan), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a TWIK-9 protein is preferably replaced with another amino acid residue from the same side chain 10 family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a TWIK-9 coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for TWIK-9 biological activity to identify mutants that retain activity. Following mutagenesis of SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid deposited with ATCC as Accession Number 15 \_\_\_\_\_, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In a preferred embodiment, a mutant TWIK-9 protein can be assayed for the ability to: (1) interact with a non-TWIK protein molecule; (2) activate a TWIK-dependent signal transduction pathway; (3) modulate the release of neurotransmitters; 20 (4) modulating membrane excitability; (5) influence the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation, (6) modulate processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials, and (7) mediate nociception.

25 In addition to the nucleic acid molecules encoding TWIK-9 proteins described above, another aspect of the invention pertains to isolated nucleic acid molecules which are antisense thereto. In an exemplary embodiment, the invention provides an isolated nucleic acid molecule which is antisense to a TWIK-9 nucleic acid molecule (*e.g.*, is antisense to the coding strand of a TWIK-9 nucleic acid molecule). An "antisense" 30 nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid. The antisense nucleic acid can be complementary to an entire TWIK-9 coding strand, or to only a portion 35 thereof. In one embodiment, an antisense nucleic acid molecule is antisense to "coding region sequences" of the coding strand of a nucleotide sequence encoding TWIK-9. The term "coding region sequences" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues (*e.g.*, the coding region

sequences of human TWIK-9 corresponding to SEQ ID NO:3). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding TWIK-9. The term "noncoding region" refers to 5' and/or 3' sequences which flank the coding region sequences that are not translated into amino acids (also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding TWIK-9 disclosed herein (*e.g.*, SEQ ID NO:3), antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to coding region sequences of TWIK-9 mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the TWIK-9 mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a TWIK-9 protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by  
5 conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be  
10 modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to  
15 cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention  
20 is an  $\alpha$ -anomeric nucleic acid molecule. An  $\alpha$ -anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual  $\beta$ -units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids Res.* 15:6625-6641). The antisense nucleic acid molecule can also comprise a 2'-*o*-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res.* 15:6131-6148) or a  
25 chimeric RNA-DNA analogue (Inoue *et al.* (1987) *FEBS Lett.* 215:327-330).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes  
30 (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave TWIK-9 mRNA transcripts to thereby inhibit translation of TWIK-9 mRNA. A ribozyme having specificity for a TWIK-9-encoding nucleic acid can be designed based upon the nucleotide sequence of a TWIK-9 cDNA disclosed herein (*i.e.*, SEQ ID NO:1 or 3, or the nucleotide sequence of the DNA insert of the plasmid  
35 deposited with ATCC as Accession Number \_\_\_\_\_). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a TWIK-9-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S.

Patent No. 5,116,742. Alternatively, TWIK-9 mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel, D. and Szostak, J.W. (1993) *Science* 261:1411-1418.

Alternatively, TWIK-9 gene expression can be inhibited by targeting nucleotide  
5 sequences complementary to the regulatory region of the TWIK-9 (*e.g.*, the TWIK-9 promoter and/or enhancers; *e.g.*, nucleotides 1-14 of SEQ ID NO:1) to form triple helical structures that prevent transcription of the TWIK-9 gene in target cells. See generally, Helene, C. (1991) *Anticancer Drug Des.* 6(6):569-84; Helene, C. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher, L.J. (1992) *Bioassays* 14(12):807-15.

10 In yet another embodiment, the TWIK-9 nucleic acid molecules of the present invention can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup, B. *et al.* (1996) *Bioorganic & Medicinal*  
15 *Chemistry* 4(1):5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The  
20 synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup, B. *et al.* (1996) *supra*; Perry-O'Keefe *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14670-675.

PNAs of TWIK-9 nucleic acid molecules can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents  
25 for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of TWIK-9 nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as 'artificial restriction enzymes' when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup, B. *et al.* (1996) *supra*)); or  
30 as probes or primers for DNA sequencing or hybridization (Hyrup, B. *et al.* (1996) *supra*; Perry-O'Keefe *et al.* (1996) *supra*).

In another embodiment, PNAs of TWIK-9 can be modified, (*e.g.*, to enhance their stability or cellular uptake), by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques  
35 of drug delivery known in the art. For example, PNA-DNA chimeras of TWIK-9 nucleic acid molecules can be generated which may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes (*e.g.*, RNase H and DNA polymerases) to interact with the DNA portion while the PNA



- portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup, B. *et al.* (1996) *supra*). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup, B. *et al.* (1996) *supra* and Finn, P.J. *et al.* (1996) *Nucleic Acids Res.* 24 (17):3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry and modified nucleoside analogs, *e.g.*, 5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used as a between the PNA and the 5' end of DNA (Mag, M. *et al.* (1989) *Nucleic Acid Res.* 17:5973-88).
- 10 PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn, P.J. *et al.* (1996) *supra*). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment (Peterser, K.H. *et al.* (1975) *Bioorganic Med. Chem. Lett.* 5: 1119-11124).
- 15 In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:6553-6556; Lemaitre *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication
- 20 No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al.* (1988) *Bio-Techniques* 6:958-976) or intercalating agents (See, *e.g.*, Zon (1988) *Pharm. Res.* 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).
- 25 Alternatively, the expression characteristics of an endogenous TWIK-9 gene within a cell line or microorganism may be modified by inserting a heterologous DNA regulatory element into the genome of a stable cell line or cloned microorganism such that the inserted regulatory element is operatively linked with the endogenous TWIK-9 gene. For example, an endogenous TWIK-9 gene which is normally "transcriptionally
- 30 silent", *i.e.*, a TWIK-9 gene which is normally not expressed, or is expressed only at very low levels in a cell line or microorganism, may be activated by inserting a regulatory element which is capable of promoting the expression of a normally expressed gene product in that cell line or microorganism. Alternatively, a transcriptionally silent, endogenous TWIK-9 gene may be activated by insertion of a
- 35 promiscuous regulatory element that works across cell types.

A heterologous regulatory element may be inserted into a stable cell line or cloned microorganism, such that it is operatively linked with an endogenous TWIK-9 gene, using techniques, such as targeted homologous recombination, which are well

known to those of skill in the art, and described, *e.g.*, in Chappel, U.S. Patent No. 5,272,071; PCT publication No. WO 91/06667, published May 16, 1991.

## II. Isolated TWIK-9 Proteins and Anti-TWIK-9 Antibodies

5           One aspect of the invention pertains to isolated or recombinant TWIK-9 proteins and polypeptides, and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise anti-TWIK-9 antibodies. In one embodiment, native TWIK-9 proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In  
10 another embodiment, TWIK-9 proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a TWIK-9 protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

          An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or  
15 tissue source from which the TWIK-9 protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of TWIK-9 protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially  
20 free of cellular material" includes preparations of TWIK-9 protein having less than about 30% (by dry weight) of non-TWIK-9 protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-TWIK-9 protein, still more preferably less than about 10% of non-TWIK-9 protein, and most preferably less than about 5% non-TWIK-9 protein. When the TWIK-9 protein or biologically  
25 active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

          The language "substantially free of chemical precursors or other chemicals"  
30 includes preparations of TWIK-9 protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of TWIK-9 protein having less than about 30% (by dry weight) of chemical precursors or non-TWIK-9 chemicals, more preferably less  
35 than about 20% chemical precursors or non-TWIK-9 chemicals, still more preferably less than about 10% chemical precursors or non-TWIK-9 chemicals, and most preferably less than about 5% chemical precursors or non-TWIK-9 chemicals.

As used herein, a "biologically active portion" of a TWIK-9 protein includes a fragment of a TWIK-9 protein which participates in an interaction between a TWIK-9 molecule and a non-TWIK-9 molecule (*e.g.*, a TWIK-9 ligand or substrate).

Biologically active portions of a TWIK-9 protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the TWIK-9 amino acid sequences, *e.g.*, the amino acid sequences shown in SEQ ID NO:2, which include sufficient amino acid residues to exhibit at least one activity of a TWIK-9 protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the TWIK-9 protein, *e.g.*, modulation of pain signaling mechanisms, membrane excitability or neurotransmitter release. A biologically active portion of a TWIK-9 protein can be a polypeptide which is, for example, 10, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300 or more amino acids in length. Biologically active portions of a TWIK-9 protein can be used as targets for developing agents which modulate a TWIK-9 mediated activity, *e.g.*, modulation of pain signaling mechanisms, membrane excitability or neurotransmitter release activities.

In one embodiment, a biologically active portion of a TWIK-9 protein comprises at least one preferably two, three, or four transmembrane domains, and/or at least one, preferably two pore loops (P-loops). Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native TWIK-9 protein.

Another aspect of the invention features fragments of the protein having the amino acid sequence of SEQ ID NO:2, for example, for use as immunogens. In one embodiment, a fragment comprises at least 5 amino acids (*e.g.*, contiguous or consecutive amino acids) of the amino acid sequence of SEQ ID NO:2, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with the ATCC as Accession Number \_\_\_\_\_. In another embodiment, a fragment comprises at least 10, 15, 20, 25, 30, 35, 40, 45, 50 or more amino acids (*e.g.*, contiguous or consecutive amino acids) of the amino acid sequence of SEQ ID NO:2, or an amino acid sequence encoded by the DNA insert of the plasmid deposited with the ATCC as Accession Number \_\_\_\_\_.

In a preferred embodiment, a TWIK-9 protein has an amino acid sequence shown in SEQ ID NO:2. In other embodiments, the TWIK-9 protein is substantially identical to SEQ ID NO:2, and retains the functional activity of the protein of SEQ ID NO:2, yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail in subsection I above. In another embodiment, the TWIK-9 protein is a protein which comprises an amino acid sequence at least about 50%, 55%,

60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to SEQ ID NO:2.

In another embodiment, the invention features a TWIK-9 protein which is encoded by a nucleic acid molecule consisting of a nucleotide sequence at least about  
5 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identical to a nucleotide sequence of SEQ ID NO:1 or 3, or a complement thereof. This invention further features a TWIK-9 protein which is encoded by a nucleic acid molecule consisting of a nucleotide sequence which hybridizes under stringent hybridization conditions to a complement of a nucleic acid  
10 molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3, or a complement thereof.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid  
15 sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (*e.g.*, when aligning a  
20 second sequence to the TWIK-9 amino acid sequence of SEQ ID NO:2 having 374 amino acid residues, at least 112, preferably at least 150, more preferably at least 187, even more preferably at least 224, and even more preferably at least 262, 299 or 337 amino acid residues are aligned). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a  
25 position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into  
30 account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using  
35 the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet

another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A preferred, non-limiting example of  
5 parameters to be used in conjunction with the GAP program include a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of Meyers and Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988)) which has been incorporated into the ALIGN program  
10 (version 2.0 or version 2.0U), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example,  
15 identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to TWIK-9 nucleic acid molecules of the invention. BLAST protein  
20 searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to TWIK-9 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the  
25 respective programs (*e.g.*, XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

The invention also provides TWIK-9 chimeric or fusion proteins. As used herein, a TWIK-9 "chimeric protein" or "fusion protein" comprises a TWIK-9 polypeptide operatively linked to a non-TWIK-9 polypeptide. A "TWIK-9 polypeptide"  
30 refers to a polypeptide having an amino acid sequence corresponding to TWIK-9, whereas a "non-TWIK-9 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the TWIK-9 protein, *e.g.*, a protein which is different from the TWIK-9 protein and which is derived from the same or a different organism. Within a TWIK-9 fusion protein the  
35 TWIK-9 polypeptide can correspond to all or a portion of a TWIK-9 protein. In a preferred embodiment, a TWIK-9 fusion protein comprises at least one biologically active portion of a TWIK-9 protein. In another preferred embodiment, a TWIK-9 fusion protein comprises at least two biologically active portions of a TWIK-9 protein. Within

the fusion protein, the term "operatively linked" is intended to indicate that the TWIK-9 polypeptide and the non-TWIK-9 polypeptide are fused in-frame to each other. The non-TWIK-9 polypeptide can be fused to the N-terminus or C-terminus of the TWIK-9 polypeptide.

- 5 For example, in one embodiment, the fusion protein is a GST-TWIK-9 fusion protein in which the TWIK-9 sequences are fused to the C-terminus of the GST sequences. Such fusion proteins can facilitate the purification of recombinant TWIK-9. In another embodiment, the fusion protein is a TWIK-9 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian  
10 host cells), expression and/or secretion of TWIK-9 can be increased through use of a heterologous signal sequence.

- The TWIK-9 fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. The TWIK-9 fusion proteins can be used to affect the bioavailability of a TWIK-9 ligand and/or substrate.  
15 Use of TWIK-9 fusion proteins may be useful therapeutically for the treatment of disorders caused by, for example, (i) aberrant modification or mutation of a gene encoding a TWIK-9 protein; (ii) mis-regulation of the TWIK-9 gene; and (iii) aberrant post-translational modification of a TWIK-9 protein.

- Moreover, the TWIK-9-fusion proteins of the invention can be used as  
20 immunogens to produce anti-TWIK-9 antibodies in a subject, to purify TWIK-9 substrates, and in screening assays to identify molecules which inhibit or enhance the interaction of TWIK-9 with a TWIK-9 ligand and/or substrate.

- Preferably, a TWIK-9 chimeric or fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the  
25 different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene  
30 can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel  
35 *et al.* John Wiley & Sons:1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A TWIK-9-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the TWIK-9 protein.

The present invention also pertains to variants of the TWIK-9 proteins which function as either TWIK-9 agonists (mimetics) or as TWIK-9 antagonists. Variants of the TWIK-9 proteins can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of a TWIK-9 protein. An agonist of the TWIK-9 proteins can retain  
5 substantially the same, or a subset, of the biological activities of the naturally occurring form of a TWIK-9 protein. An antagonist of a TWIK-9 protein can inhibit one or more of the activities of the naturally occurring form of the TWIK-9 protein by, for example, competitively modulating a TWIK-9-mediated activity of a TWIK-9 protein. Thus, specific biological effects can be elicited by treatment with a variant of limited function.  
10 In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the TWIK-9 protein.

In one embodiment, variants of a TWIK-9 protein which function as either  
15 TWIK-9 agonists (mimetics) or as TWIK-9 antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of a TWIK-9 protein for TWIK-9 protein agonist or antagonist activity. In one embodiment, a variegated library of TWIK-9 variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of TWIK-9 variants  
20 can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential TWIK-9 sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of TWIK-9 sequences therein. There are a variety of methods which can be used to produce libraries of potential  
25 TWIK-9 variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential TWIK-9 sequences. Methods for synthesizing degenerate  
30 oligonucleotides are known in the art (see, *e.g.*, Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu. Rev. Biochem.* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucleic Acid Res.* 11:477).

In addition, libraries of fragments of a TWIK-9 protein coding sequence can be used to generate a variegated population of TWIK-9 fragments for screening and  
35 subsequent selection of variants of a TWIK-9 protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a TWIK-9 coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA,

renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which  
5 encodes N-terminal, C-terminal and internal fragments of various sizes of the TWIK-9 protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for  
10 rapid screening of the gene libraries generated by the combinatorial mutagenesis of TWIK-9 proteins. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in  
15 which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify TWIK-9 variants (Arkin and Yourvan (1992) *Proc. Natl. Acad. Sci. USA* 89:7811-7815; Delgrave *et al.* (1993) *Protein*  
20 *Engineering* 6(3):327-331).

In one embodiment, cell based assays can be exploited to analyze a variegated TWIK-9 library. For example, a library of expression vectors can be transfected into a cell line, *e.g.*, a neuronal cell line, which ordinarily responds to TWIK-9 in a particular TWIK-9 ligand-dependent manner. The transfected cells are then contacted with TWIK-  
25 9 and the effect of the expression of the mutant on, *e.g.*, membrane excitability of TWIK-9 can be detected. Plasmid DNA can then be recovered from the cells which score for inhibition, or alternatively, potentiation of signaling by the TWIK-9 ligand, and the individual clones further characterized.

An isolated TWIK-9 protein, or a portion or fragment thereof, can be used as an  
30 immunogen to generate antibodies that bind TWIK-9 using standard techniques for polyclonal and monoclonal antibody preparation. A full-length TWIK-9 protein can be used or, alternatively, the invention provides antigenic peptide fragments of TWIK-9 for use as immunogens. The antigenic peptide of TWIK-9 comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO:2 and encompasses an  
35 epitope of TWIK-9 such that an antibody raised against the peptide forms a specific immune complex with TWIK-9. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more



preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues.

Preferred epitopes encompassed by the antigenic peptide are regions of TWIK-9 that are located on the surface of the protein, *e.g.*, hydrophilic regions, as well as regions with high antigenicity (see, for example, Figure 2).

A TWIK-9 immunogen typically is used to prepare antibodies by immunizing a suitable subject (*e.g.*, rabbit, goat, mouse, or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, recombinantly expressed TWIK-9 protein or a chemically-synthesized TWIK-9 polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic TWIK-9 preparation induces a polyclonal anti-TWIK-9 antibody response.

Accordingly, another aspect of the invention pertains to anti-TWIK-9 antibodies. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as TWIK-9. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and F(ab')<sub>2</sub> fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies that bind TWIK-9. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of TWIK-9. A monoclonal antibody composition thus typically displays a single binding affinity for a particular TWIK-9 protein with which it immunoreacts.

Polyclonal anti-TWIK-9 antibodies can be prepared as described above by immunizing a suitable subject with a TWIK-9 immunogen. The anti-TWIK-9 antibody titer in the immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized TWIK-9. If desired, the antibody molecules directed against TWIK-9 can be isolated from the mammal (*e.g.*, from the blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction. At an appropriate time after immunization, *e.g.*, when the anti-TWIK-9 antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975) *Nature* 256:495-497 (see also Brown *et al.* (1981) *J. Immunol.* 127:539-46; Brown *et al.* (1980) *J. Biol. Chem.* 255:4980-83; Yeh *et al.*

- (1976) *Proc. Natl. Acad. Sci. USA* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985), *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques.
- 5 The technology for producing monoclonal antibody hybridomas is well known (see generally Kenneth, R. H. in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); Lerner, E. A. (1981) *Yale J. Biol. Med.*, 54:387-402; Gefter, M. L. *et al.* (1977) *Somatic Cell Genet.* 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes
- 10 (typically splenocytes) from a mammal immunized with a TWIK-9 immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds TWIK-9.

- Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-TWIK-9
- 15 monoclonal antibody (see, *e.g.*, Galfre, G. *et al.* (1977) *Nature* 266:55052; Gefter *et al.* *Somatic Cell Genet.*, *supra*; Lerner (1981) *supra*; Kenneth, *Monoclonal Antibodies*, *supra*). Moreover, the ordinarily skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (*e.g.*, a myeloma cell line) is derived from the same mammalian species as the
- 20 lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines can be used as a
- 25 fusion partner according to standard techniques, *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from ATCC. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively fused myeloma
- 30 cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind TWIK-9, *e.g.*, using a standard ELISA assay.

- Alternative to preparing monoclonal antibody-secreting hybridomas, a
- 35 monoclonal anti-TWIK-9 antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with TWIK-9 to thereby isolate immunoglobulin library members that bind TWIK-9. Kits for generating and screening phage display libraries are commercially

available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner  
5 *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT International Publication No. WO 92/18619; Dower *et al.* PCT International Publication No. WO 91/17271; Winter *et al.* PCT International Publication WO 92/20791; Markland *et al.* PCT International Publication No. WO 92/15679; Breitling *et al.* PCT International Publication WO 93/01288; McCafferty *et al.* PCT International Publication No. WO 92/01047; Garrard  
10 *et al.* PCT International Publication No. WO 92/09690; Ladner *et al.* PCT International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J.* 12:725-734; Hawkins *et al.* (1992) *J. Mol. Biol.* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *Proc. Natl.*  
15 *Acad. Sci. USA* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc. Acid Res.* 19:4133-4137; Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; and McCafferty *et al.* (1990) *Nature* 348:552-554.

Additionally, recombinant anti-TWIK-9 antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions,  
20 which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Application No. PCT/US86/02269; Akira, *et al.* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496;  
25 Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT International Publication No. WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *Proc. Natl. Acad. Sci. USA*  
30 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl. Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

35 An anti-TWIK-9 antibody (e.g., monoclonal antibody) can be used to isolate TWIK-9 by standard techniques; such as affinity chromatography or immunoprecipitation. An anti-TWIK-9 antibody can facilitate the purification of natural TWIK-9 from cells and of recombinantly produced TWIK-9 expressed in host cells.

Moreover, an anti-TWIK-9 antibody can be used to detect TWIK-9 protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the TWIK-9 protein. Anti-TWIK-9 antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for  
5 example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline  
10 phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent  
15 materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

### III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors, for example recombinant  
20 expression vectors, containing a TWIK-9 nucleic acid molecule or vectors containing a nucleic acid molecule which encodes a TWIK-9 protein (or a portion thereof). As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA  
25 segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host  
30 cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can  
35 be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is  
5 operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory  
10 sequence" is intended to include promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Methods Enzymol.* 185:3-7. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in certain  
15 host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by  
20 nucleic acids as described herein (*e.g.*, TWIK-9 proteins, mutant forms of TWIK-9 proteins, fusion proteins, and the like).

Accordingly, an exemplary embodiment provides a method for producing a protein, preferably a TWIK-9 protein, by culturing in a suitable medium a host cell of the invention (*e.g.*, a mammalian host cell such as a non-human mammalian cell)  
25 containing a recombinant expression vector, such that the protein is produced.

The recombinant expression vectors of the invention can be designed for expression of TWIK-9 proteins in prokaryotic or eukaryotic cells. For example, TWIK-9 proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are  
30 discussed further in Goeddel (1990) *supra*. Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either  
35 fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification

of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Purified fusion proteins can be utilized in TWIK-9 activity assays, (e.g., direct assays or competitive assays described in detail below), or to generate antibodies specific for TWIK-9 proteins, for example. In a preferred embodiment, a TWIK-9 fusion protein expressed in a retroviral expression vector of the present invention can be utilized to infect bone marrow cells, which are subsequently transplanted into irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (e.g., six (6) weeks).

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.* (1990) *Methods Enzymol.* 185:60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S. (1990) *Methods Enzymol.* 185:119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.* (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the TWIK-9 expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.* (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.* (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp., San Diego, CA).

Alternatively, TWIK-9 proteins can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 5 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the 10 expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2<sup>nd</sup>, ed., Cold Spring Harbor Laboratory, Cold Spring* 15 *Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.*

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable 20 tissue-specific promoters include the alpha-myosin heavy chain promoter (cardiac specific), albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore 25 (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), adipose-specific promoters (U.S. Patent No. 5,476,926; WO 92/06104), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication 30 No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the  $\alpha$ -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense 35 orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to TWIK-9 mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen

which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, "Antisense RNA as a molecular tool for genetic analysis", *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a TWIK-9 nucleic acid molecule of the invention is introduced, *e.g.*, a TWIK-9 nucleic acid molecule within a vector (*e.g.*, a recombinant expression vector) or a TWIK-9 nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, a TWIK-9 protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup>, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred



selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a TWIK-9 protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) a TWIK-9 protein. Accordingly, the invention further provides methods for producing a TWIK-9 protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of the invention (into which a recombinant expression vector encoding a TWIK-9 protein has been introduced) in a suitable medium such that a TWIK-9 protein is produced. In another embodiment, the method further comprises isolating a TWIK-9 protein from the medium or the host cell.

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which TWIK-9-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous TWIK-9 sequences have been introduced into their genome or homologous recombinant animals in which endogenous TWIK-9 sequences have been altered. Such animals are useful for studying the function and/or activity of a TWIK-9 protein and for identifying and/or evaluating modulators of TWIK-9 activity. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens, amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous TWIK-9 gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing a TWIK-9-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection or retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The TWIK-9 cDNA sequence of SEQ ID NO:1

can be introduced as a transgene into the genome of a non-human animal. Alternatively, a non-human homologue of a human TWIK-9 gene, such as a rat or mouse TWIK-9 gene, can be used as a transgene. Alternatively, a TWIK-9 gene homologue, such as another TWIK-9 family member, can be isolated based on hybridization to the TWIK-9 cDNA sequences of SEQ ID NO:1 or 3, or the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_ (described further in subsection I above) and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to a TWIK-9 transgene to direct expression of a TWIK-9 protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of a TWIK-9 transgene in its genome and/or expression of TWIK-9 mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding a TWIK-9 protein can further be bred to other transgenic animals carrying other transgenes.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a TWIK-9 gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the TWIK-9 gene. The TWIK-9 gene can be a human gene (*e.g.*, the cDNA of SEQ ID NO:3), but more preferably, is a non-human homologue of a human TWIK-9 gene (*e.g.*, a cDNA isolated by stringent hybridization with the nucleotide sequence of SEQ ID NO:1). For example, a mouse TWIK-9 gene can be used to construct a homologous recombination nucleic acid molecule, *e.g.*, a vector, suitable for altering an endogenous TWIK-9 gene in the mouse genome. In a preferred embodiment, the homologous recombination nucleic acid molecule is designed such that, upon homologous recombination, the endogenous TWIK-9 gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the homologous recombination nucleic acid molecule can be designed such that, upon homologous recombination, the endogenous TWIK-9 gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous TWIK-9 protein). In the homologous recombination nucleic acid molecule, the altered portion of the TWIK-9 gene is flanked at its 5' and 3'

ends by additional nucleic acid sequence of the TWIK-9 gene to allow for homologous recombination to occur between the exogenous TWIK-9 gene carried by the homologous recombination nucleic acid molecule and an endogenous TWIK-9 gene in a cell, *e.g.*, an embryonic stem cell. The additional flanking TWIK-9 nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the homologous recombination nucleic acid molecule (see, *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a description of homologous recombination vectors). The homologous recombination nucleic acid molecule is introduced into a cell, *e.g.*, an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced TWIK-9 gene has homologously recombined with the endogenous TWIK-9 gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells can then be injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, Robertson, E. J. ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination nucleic acid molecules, *e.g.*, vectors, or homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In another embodiment, transgenic non-humans animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, see, *e.g.*, Lakso *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) *Science* 251:1351-1355). If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. *et al.* (1997) *Nature* 385:810-813 and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and  
5 induced to exit the growth cycle and enter G<sub>0</sub> phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal  
10 will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

#### IV. Pharmaceutical Compositions

The TWIK-9 nucleic acid molecules, TWIK-9 proteins, fragments thereof, anti-TWIK-9 antibodies, and TWIK-9 modulators (also referred to herein as “active  
15 compounds”) of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language “pharmaceutically acceptable carrier” is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and  
20 absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

25 A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following  
30 components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of  
35 tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS).  
5 In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various  
10 antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.  
20

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a fragment of a TWIK-9 protein or an anti-TWIK-9 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions  
25 are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.  
30

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. oral compositions can also be prepared  
35 using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the

following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a  
5 sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

10 Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the  
15 use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention  
20 enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate,  
25 polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically  
30 acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject  
35 to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound

and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for  
5 determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a  
10 delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little  
15 or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of  
20 the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

As defined herein, a therapeutically effective amount of protein or polypeptide  
25 (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, preferably about 0.01 to 25 mg/kg body weight, more preferably about 0.1 to 20 mg/kg body weight, and even more preferably about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The skilled artisan will appreciate that certain factors may influence the dosage required to effectively treat a subject, including but not  
30 limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

In a preferred example, a subject is treated with antibody, protein, or polypeptide  
35 in the range of between about 0.1 to 20 mg/kg body weight, one time per week for between about 1 to 10 weeks, preferably between 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. It will also be appreciated that the effective dosage of antibody, protein, or polypeptide used for

treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein.

The present invention encompasses agents which modulate expression or activity. An agent may, for example, be a small molecule. For example, such small molecules include, but are not limited to, peptides, peptidomimetics, amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds. It is understood that appropriate doses of small molecule agents depends upon a number of factors within the ken of the ordinarily skilled physician, veterinarian, or researcher. The dose(s) of the small molecule will vary, for example, depending upon the identity, size, and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered, if applicable, and the effect which the practitioner desires the small molecule to have upon the nucleic acid or polypeptide of the invention.

Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (*e.g.*, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. Such appropriate doses may be determined using the assays described herein. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.



In certain embodiments of the invention, a modulator of TWIK-9 activity is administered in combination with other agents (*e.g.*, a small molecule), or in conjunction with another, complementary treatment regime. For example, in one embodiment, a modulator of TWIK-9 activity is used to treat a central nervous system disorder.

- 5 Accordingly, modulation of TWIK-9 activity may be used in conjunction with, for example, neuroactive and/or neurotrophic agents.

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include  
10 taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites  
15 (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and  
20 doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical  
25 therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, alpha-interferon, beta-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers  
30 such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, *e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs  
35 In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer

Therapy: A Review", in *Monoclonal Antibodies '84: Biological And Clinical Applications*, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in *Monoclonal Antibodies For Cancer Detection And Therapy*, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", *Immunol. Rev.*, 62:119-58 (1982).  
Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

#### V. Uses and Methods of the Invention

The nucleic acid molecules, proteins, protein homologues, protein fragments, antibodies, peptides, peptidomimetics, and small molecules described herein can be used in one or more of the following methods: a) screening assays; b) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics); and c) methods of treatment (*e.g.*, therapeutic and prophylactic). As described herein, a TWIK-9 protein of the invention has one or more of the following activities: (1) interacting with a non-TWIK protein molecule; (2) activating a TWIK-dependent signal transduction pathway; (3) modulating the release of neurotransmitters; (4) modulating membrane excitability; (5) influencing the resting potential of membranes, wave forms and frequencies of action potentials, and thresholds of excitation, (6) modulating processes which underlie learning and memory, such as integration of sub-threshold synaptic responses and the conductance of back-propagating action potentials, and (7) mediating nociception.

The isolated nucleic acid molecules of the invention can be used, for example, to express TWIK-9 protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect TWIK-9 mRNA (*e.g.*, in a biological sample) or a

genetic alteration in a TWIK-9 gene, and to modulate TWIK-9 activity, as described further below. The TWIK-9 proteins can be used to treat disorders characterized by insufficient or excessive production of a TWIK-9 ligand or substrate, or production of TWIK-9 inhibitors, for example, potassium channel associated disorders.

- 5           As used interchangeably herein, an “potassium channel-associated disorder” or a “TWIK-9-associated disorder” includes a disorder, disease or condition which is caused or characterized by a misregulation (*e.g.*, downregulation or upregulation) of a potassium channel activity or a TWIK-9-mediated activity. Potassium channel-associated disorders can detrimentally affect cellular functions such as cellular  
10 proliferation, growth, differentiation, and/or migration; inter- or intra-cellular communication; tissue function, such as muscular function or cardiac function; systemic responses in an organism, such as nervous system responses.

- Examples of potassium channel associated disorders include central nervous system (CNS) disorders such as cognitive and neurodegenerative disorders, examples of  
15 which include, but are not limited to, Alzheimer’s disease, dementias related to Alzheimer’s disease (such as Pick’s disease), Parkinson’s and other Lewy diffuse body diseases, senile dementia, Huntington’s disease, Gilles de la Tourette’s syndrome, multiple sclerosis, amyotrophic lateral sclerosis, movement disorders, progressive supranuclear palsy, epilepsy, AIDS related dementia, and Jakob-Creutzfeldt disease;  
20 autonomic function disorders such as hypertension and sleep disorders, and neuropsychiatric disorders, such as depression, schizophrenia, schizoaffective disorder, korsakoff’s psychosis, mania, anxiety disorders, or phobic disorders; learning or memory disorders, *e.g.*, amnesia or age-related memory loss, attention deficit disorder, dysthymic disorder, major depressive disorder, mania, obsessive-compulsive disorder,  
25 psychoactive substance use disorders, anxiety, phobias, panic disorder, as well as bipolar affective disorder, *e.g.*, severe bipolar affective (mood) disorder (BP-1), and bipolar affective neurological disorders, *e.g.*, migraine and obesity. Further CNS-related disorders include, for example, those listed in the American Psychiatric Association’s Diagnostic and Statistical manual of Mental Disorders (DSM), the most current version  
30 of which is incorporated herein by reference in its entirety.

- Further examples of potassium channel associated disorders include cardiac-related disorders. Cardiovascular system disorders in which the TWIK-9 molecules of the invention may be directly or indirectly involved include arteriosclerosis, ischemia reperfusion injury, restenosis, arterial inflammation, vascular wall remodeling,  
35 ventricular remodeling, rapid ventricular pacing, coronary microembolism, tachycardia, bradycardia, pressure overload, aortic bending, coronary artery ligation, vascular heart disease, atrial fibrillation, Jervell syndrome, Lange-Nielsen syndrome, long-QT syndrome, congestive heart failure, sinus node dysfunction, angina, heart failure,

hypertension, atrial fibrillation, atrial flutter, dilated cardiomyopathy, idiopathic cardiomyopathy, myocardial infarction, coronary artery disease, cerebral ischemia, stroke, coronary artery spasm, and arrhythmia. TWIK-9-mediated or related disorders also include disorders of the musculoskeletal system such as paralysis and muscle  
5 weakness, *e.g.*, ataxia, myotonia, and myokymia.

Further disorders in which the TWIK-9 molecules of the invention may be involved are pain disorders. Pain disorders include those that affect pain signaling mechanisms. As used herein, the term "pain signaling mechanisms" includes the cellular mechanisms involved in the development and regulation of pain, *e.g.*, pain elicited by  
10 noxious chemical, mechanical, or thermal stimuli, in a subject, *e.g.*, a mammal such as a human. In mammals, the initial detection of noxious chemical, mechanical, or thermal stimuli, a process referred to as "nociception", occurs predominantly at the peripheral terminals of specialized, small diameter sensory neurons. These sensory neurons transmit the information to the central nervous system, evoking a perception of pain or  
15 discomfort and initiating appropriate protective reflexes. The TWIK-9 molecules of the present invention may be present on these sensory neurons and, thus, may be involved in detecting these noxious chemical, mechanical, or thermal stimuli and transducing this information into membrane depolarization events. Thus, the TWIK-9 molecules by participating in pain signaling mechanisms, may modulate pain elicitation and act as  
20 targets for developing novel diagnostic targets and therapeutic agents to control pain. Examples of pain disorders include headache (*e.g.*, tension headache or migraine), back pain, cancer pain, arthritis pain, or neurogenic pain.

Potassium channel disorders also include cellular proliferation, growth, differentiation, or migration disorders. Cellular proliferation, growth, differentiation, or  
25 migration disorders include those disorders that affect cell proliferation, growth, differentiation, or migration processes. As used herein, a "cellular proliferation, growth, differentiation, or migration process" is a process by which a cell increases in number, size or content, by which a cell develops a specialized set of characteristics which differ from that of other cells, or by which a cell moves closer to or further from a particular  
30 location or stimulus. The TWIK-9 molecules of the present invention are involved in signal transduction mechanisms, which are known to be involved in cellular growth, differentiation, and migration processes. Thus, the TWIK-9 molecules may modulate cellular growth, differentiation, or migration, and may play a role in disorders characterized by aberrantly regulated growth, differentiation, or migration. Such  
35 disorders include cancer, *e.g.*, carcinoma, sarcoma, lymphoma or leukemia, examples of which include, but are not limited to, breast, endometrial, ovarian, uterine, hepatic, gastrointestinal, prostate, colorectal, and lung cancer, melanoma, neurofibromatosis, adenomatous polyposis of the colon, Wilms' tumor, nephroblastoma, teratoma,

rhabdomyosarcoma; tumor invasion, angiogenesis and metastasis; skeletal dysplasia; neuronal deficiencies resulting from impaired neural induction and patterning; and hematopoietic and/or myeloproliferative disorders.

TWIK-9-associated or related disorders also include disorders affecting tissues  
5 in which TWIK-9 protein is expressed.

In addition, the TWIK-9 proteins can be used to screen for naturally occurring TWIK-9 ligands or substrates, to screen for drugs or compounds which modulate TWIK-9 activity, as well as to treat disorders characterized by insufficient or excessive production of TWIK-9 protein or production of TWIK-9 protein forms which have  
10 decreased, aberrant or unwanted activity compared to TWIK-9 wild type protein (e.g., potassium channel associated disorders).

Moreover, the anti-TWIK-9 antibodies of the invention can be used to detect and isolate TWIK-9 proteins, regulate the bioavailability of TWIK-9 proteins, and modulate TWIK-9 activity.

15

A. Screening Assays:

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (e.g., peptides, peptidomimetics, small molecules or other drugs) which bind to TWIK-9 proteins, have  
20 a stimulatory or inhibitory effect on, for example, TWIK-9 expression or TWIK-9 activity, or have a stimulatory or inhibitory effect on, for example, the expression or activity of a TWIK-9 ligand or substrate.

In one embodiment, the invention provides assays for screening candidate or test compounds which are ligands or substrates of a TWIK-9 protein or polypeptide or  
25 biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of a TWIK-9 protein or polypeptide or biologically active portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological  
30 libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds  
35 (Lam, K.S. (1997) *Anticancer Drug Des.* 12:45).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example, in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med.*

*Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In one embodiment, an assay is a cell-based assay in which a cell which expresses a TWIK-9 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate TWIK-9 activity is determined. Determining the ability of the test compound to modulate TWIK-9 activity can be accomplished by monitoring, for example, potassium current, neurotransmitter release and/or membrane excitability in a cell which expresses TWIK-9. The cell, for example, can be of mammalian origin, *e.g.*, a neuronal cell.

The ability of the test compound to modulate TWIK-9 binding to a ligand or substrate, or to bind to TWIK-9 can also be determined. Determining the ability of the test compound to modulate TWIK-9 binding to a ligand or substrate can be accomplished, for example, by coupling the TWIK-9 ligand or substrate with a radioisotope or enzymatic label such that binding of the TWIK-9 ligand or substrate to TWIK-9 can be determined by detecting the labeled TWIK-9 ligand or substrate in a complex. Alternatively, TWIK-9 could be coupled with a radioisotope or enzymatic label to monitor the ability of a test compound to modulate TWIK-9 binding to a TWIK-9 ligand or substrate in a complex. Determining the ability of the test compound to bind TWIK-9 can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to TWIK-9 can be determined by detecting the labeled compound in a complex. For example, compounds (*e.g.*, TWIK-9 ligands or substrates) can be labeled with  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

It is also within the scope of this invention to determine the ability of a compound (*e.g.*, a TWIK-9 ligand or substrate) to interact with TWIK-9 without the labeling of any of the interactants. For example, a microphysiometer can be used to

detect the interaction of a compound with TWIK-9 without the labeling of either the compound or the TWIK-9. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. As used herein, a "microphysiometer" (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable  
5 potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between a compound and TWIK-9.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a TWIK-9 target molecule (*e.g.*, a TWIK-9 substrate) with a test compound and determining the ability of the test compound to modulate (*e.g.* stimulate  
10 or inhibit) the activity of the TWIK-9 target molecule. Determining the ability of the test compound to modulate the activity of a TWIK-9 target molecule can be accomplished, for example, by determining the ability of the TWIK-9 protein to bind to or interact with the TWIK-9 target molecule.

Determining the ability of the TWIK-9 protein or a biologically active fragment thereof, to bind to or interact with a TWIK-9 target molecule can be accomplished by  
15 one of the methods described above for determining direct binding. In a preferred embodiment, determining the ability of the TWIK-9 protein to bind to or interact with a TWIK-9 target molecule can be accomplished by determining the activity of the target molecule. For example, the activity of the target molecule can be determined by  
20 detecting induction of a cellular response (*e.g.*, changes in intracellular K<sup>+</sup> levels), detecting catalytic/enzymatic activity of the target molecule upon an appropriate substrate, detecting the induction of a reporter gene (comprising a target-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a target-regulated cellular response.

In yet another embodiment, an assay of the present invention is a cell-free assay in which a TWIK-9 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the TWIK-9 protein or biologically active portion thereof is determined. Preferred biologically active portions of the TWIK-9 proteins to be used in assays of the present invention include fragments  
30 which participate in interactions with non-TWIK-9 molecules, *e.g.*, fragments with high surface probability scores (see, for example, Figure 2). Binding of the test compound to the TWIK-9 protein can be determined either directly or indirectly as described above. In a preferred embodiment, the assay includes contacting the TWIK-9 protein or biologically active portion thereof with a known compound which binds TWIK-9 to  
35 form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with a TWIK-9 protein, wherein determining the ability of the test compound to interact with a TWIK-9 protein

comprises determining the ability of the test compound to preferentially bind to TWIK-9 or biologically active portion thereof as compared to the known compound.

In another embodiment, the assay is a cell-free assay in which a TWIK-9 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate (*e.g.*, stimulate or inhibit) the activity of the TWIK-9 protein or biologically active portion thereof is determined. Determining the ability of the test compound to modulate the activity of a TWIK-9 protein can be accomplished, for example, by determining the ability of the TWIK-9 protein to bind to a TWIK-9 target molecule by one of the methods described above for determining direct binding. Determining the ability of the TWIK-9 protein to bind to a TWIK-9 target molecule can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In an alternative embodiment, determining the ability of the test compound to modulate the activity of a TWIK-9 protein can be accomplished by determining the ability of the TWIK-9 protein to further modulate the activity of a downstream effector of a TWIK-9 target molecule. For example, the activity of the effector molecule on an appropriate target can be determined or the binding of the effector to an appropriate target can be determined as previously described.

In yet another embodiment, the cell-free assay involves contacting a TWIK-9 protein or biologically active portion thereof with a known compound which binds the TWIK-9 protein to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the TWIK-9 protein, wherein determining the ability of the test compound to interact with the TWIK-9 protein comprises determining the ability of the TWIK-9 protein to preferentially bind to or modulate the activity of a TWIK-9 target molecule.

The cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of isolated proteins (*e.g.*, TWIK-9 proteins or biologically active portions thereof). In the case of cell-free assays in which a membrane-bound form of an isolated protein is used it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114,



Thesit<sup>®</sup>, Isotridecypoly(ethylene glycol ether)<sub>n</sub>, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

5 In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either TWIK-9 or its target molecule to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of a test compound to a TWIK-9 protein, or interaction of a TWIK-9 protein with a substrate or  
10 target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows one or both of the proteins to be bound to a matrix. For example, glutathione-S-  
15 transferase/TWIK-9 fusion proteins or glutathione-S-transferase/target fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized micrometer plates, which are then combined with the test compound or the test compound and either the non-adsorbed target protein or TWIK-9 protein, and the mixture incubated under conditions conducive to complex formation  
20 (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of TWIK-9 binding or activity determined using standard  
25 techniques.

Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example, either a TWIK-9 protein or a TWIK-9 substrate or target molecule can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated TWIK-9 protein, substrates, or target molecules can be  
30 prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with TWIK-9 protein or target molecules but which do not interfere with binding of the TWIK-9 protein to its target molecule can be derivatized to the wells of the plate, and  
35 unbound target or TWIK-9 protein trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the TWIK-9 protein or target molecule, as well as enzyme-linked assays

which rely on detecting an enzymatic activity associated with the TWIK-9 protein or target molecule.

In another embodiment, modulators of TWIK-9 expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of TWIK-9 mRNA or protein in the cell is determined. The level of expression of TWIK-9 mRNA or protein in the presence of the candidate compound is compared to the level of expression of TWIK-9 mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of TWIK-9 expression based on this comparison. For example, when expression of TWIK-9 mRNA or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of TWIK-9 mRNA or protein expression. Alternatively, when expression of TWIK-9 mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of TWIK-9 mRNA or protein expression. The level of TWIK-9 mRNA or protein expression in the cells can be determined by methods described herein for detecting TWIK-9 mRNA or protein.

In yet another aspect of the invention, the TWIK-9 proteins can be used as “bait proteins” in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696; and Brent WO94/10300) to identify other proteins which bind to or interact with TWIK-9 (“TWIK-9-binding proteins” or “TWIK-9-bp”) and are involved in TWIK-9 activity. Such TWIK-9-binding proteins are also likely to be involved in the propagation of signals by the TWIK-9 proteins or TWIK-9 targets as, for example, downstream elements of a TWIK-9-mediated signaling pathway. Alternatively, such TWIK-9-binding proteins may be TWIK-9 inhibitors.

The two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a TWIK-9 protein is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified protein (“prey” or “sample”) is fused to a gene that codes for the activation domain of the known transcription factor. If the “bait” and the “prey” proteins are able to interact, *in vivo*, forming a TWIK-9-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to

the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the TWIK-9 protein.

In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a modulating agent can be identified using a cell-based or a cell-free assay, and the ability of the agent to modulate the activity of a TWIK-9 protein can be confirmed *in vivo*, *e.g.*, in an animal such as an animal model for a neurodegenerative disorder or for pain.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, an agent identified as described herein (*e.g.*, a TWIK-9 modulating agent, an antisense TWIK-9 nucleic acid molecule, a TWIK-9-specific antibody, or a TWIK-9 binding partner) can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore, this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

## 20 B. Detection Assays

Portions or fragments of the cDNA sequences identified herein (and the corresponding complete gene sequences) can be used in numerous ways as polynucleotide reagents. For example, these sequences can be used to: (i) map their respective genes on a chromosome; and, thus, locate gene regions associated with genetic disease; (ii) identify an individual from a minute biological sample (tissue typing); and (iii) aid in forensic identification of a biological sample. These applications are described in the subsections below.

### 1. Chromosome Mapping

30 Once the sequence (or a portion of the sequence) of a gene has been isolated, this sequence can be used to map the location of the gene on a chromosome. This process is called chromosome mapping. Accordingly, portions or fragments of the TWIK-9 nucleotide sequences, described herein, can be used to map the location of the TWIK-9 genes on a chromosome. The mapping of the TWIK-9 sequences to chromosomes is an important first step in correlating these sequences with genes associated with disease.

Briefly, TWIK-9 genes can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp in length) from the TWIK-9 nucleotide sequences. Computer analysis of the TWIK-9 sequences can be used to predict primers that do not

span more than one exon in the genomic DNA, thus complicating the amplification process. These primers can then be used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the TWIK-9 sequences will yield an amplified fragment.

- 5           Somatic cell hybrids are prepared by fusing somatic cells from different mammals (*e.g.*, human and mouse cells). As hybrids of human and mouse cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. By using media in which mouse cells cannot grow, because they lack a particular enzyme, but human cells can, the one human chromosome that contains  
10 the gene encoding the needed enzyme, will be retained. By using various media, panels of hybrid cell lines can be established. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes, allowing easy mapping of individual genes to specific human chromosomes. (D'Eustachio P. *et al.* (1983) *Science* 220:919-924). Somatic cell  
15 hybrids containing only fragments of human chromosomes can also be produced by using human chromosomes with translocations and deletions.

- PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular sequence to a particular chromosome. Three or more sequences can be assigned per day using a single thermal cycler. Using the TWIK-9 nucleotide sequences  
20 to design oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes. Other mapping strategies which can similarly be used to map a TWIK-9 sequence to its chromosome include *in situ* hybridization (described in Fan, Y. *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:6223-27), pre-screening with labeled flow-sorted chromosomes, and pre-selection by hybridization to  
25 chromosome-specific cDNA libraries.

- Fluorescence *in situ* hybridization (FISH) of a DNA sequence to a metaphase chromosomal spread can further be used to provide a precise chromosomal location in one step. Chromosome spreads can be made using cells whose division has been blocked in metaphase by a chemical such as colcemid that disrupts the mitotic spindle.  
30 The chromosomes can be treated briefly with trypsin, and then stained with Giemsa. A pattern of light and dark bands develops on each chromosome, so that the chromosomes can be identified individually. The FISH technique can be used with a DNA sequence as short as 500 or 600 bases. However, clones larger than 1,000 bases have a higher likelihood of binding to a unique chromosomal location with sufficient signal intensity  
35 for simple detection. Preferably 1,000 bases, and more preferably 2,000 bases will suffice to get good results at a reasonable amount of time. For a review of this technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques (Pergamon Press, New York 1988).

Reagents for chromosome mapping can be used individually to mark a single chromosome or a single site on that chromosome, or panels of reagents can be used for marking multiple sites and/or multiple chromosomes. Reagents corresponding to noncoding regions of the genes actually are preferred for mapping purposes. Coding sequences are more likely to be conserved within gene families, thus increasing the chance of cross hybridizations during chromosomal mapping.

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. (Such data are found, for example, in McKusick, V., Mendelian Inheritance in Man, available on-line through Johns Hopkins University Welch Medical Library). The relationship between a gene and a disease, mapped to the same chromosomal region, can then be identified through linkage analysis (co-inheritance of physically adjacent genes), described in, for example, Egeland, J. *et al.* (1987) *Nature*, 325:783-787.

Moreover, differences in the DNA sequences between individuals affected and unaffected with a disease associated with the TWIK-9 gene, can be determined. If a mutation is observed in some or all of the affected individuals but not in any unaffected individuals, then the mutation is likely to be the causative agent of the particular disease. Comparison of affected and unaffected individuals generally involves first looking for structural alterations in the chromosomes, such as deletions or translocations that are visible from chromosome spreads or detectable using PCR based on that DNA sequence. Ultimately, complete sequencing of genes from several individuals can be performed to confirm the presence of a mutation and to distinguish mutations from polymorphisms.

25

## 2. Tissue Typing

The TWIK-9 sequences of the present invention can also be used to identify individuals from minute biological samples. The United States military, for example, is considering the use of restriction fragment length polymorphism (RFLP) for identification of its personnel. In this technique, an individual's genomic DNA is digested with one or more restriction enzymes, and probed on a Southern blot to yield unique bands for identification. This method does not suffer from the current limitations of "Dog Tags" which can be lost, switched, or stolen, making positive identification difficult. The sequences of the present invention are useful as additional DNA markers for RFLP (described in U.S. Patent 5,272,057).

35

Furthermore, the sequences of the present invention can be used to provide an alternative technique which determines the actual base-by-base DNA sequence of selected portions of an individual's genome. Thus, the TWIK-9 nucleotide sequences

described herein can be used to prepare two PCR primers from the 5' and 3' ends of the sequences. These primers can then be used to amplify an individual's DNA and subsequently sequence it.

Panels of corresponding DNA sequences from individuals, prepared in this  
5 manner, can provide unique individual identifications, as each individual will have a  
unique set of such DNA sequences due to allelic differences. The sequences of the  
present invention can be used to obtain such identification sequences from individuals  
and from tissue. The TWIK-9 nucleotide sequences of the invention uniquely represent  
portions of the human genome. Allelic variation occurs to some degree in the coding  
10 regions of these sequences, and to a greater degree in the noncoding regions. It is  
estimated that allelic variation between individual humans occurs with a frequency of  
about once per each 500 bases. Each of the sequences described herein can, to some  
degree, be used as a standard against which DNA from an individual can be compared  
for identification purposes. Because greater numbers of polymorphisms occur in the  
15 noncoding regions, fewer sequences are necessary to differentiate individuals. The  
noncoding sequences of SEQ ID NO:1 can comfortably provide positive individual  
identification with a panel of perhaps 10 to 1,000 primers which each yield a noncoding  
amplified sequence of 100 bases. If predicted coding sequences, such as those in SEQ  
ID NO:3 are used, a more appropriate number of primers for positive individual  
20 identification would be 500-2,000.

If a panel of reagents from TWIK-9 nucleotide sequences described herein is  
used to generate a unique identification database for an individual, those same reagents  
can later be used to identify tissue from that individual. Using the unique identification  
database, positive identification of the individual, living or dead, can be made from  
25 extremely small tissue samples.

### 3. Use of Partial TWIK-9 Sequences in Forensic Biology

DNA-based identification techniques can also be used in forensic biology.  
Forensic biology is a scientific field employing genetic typing of biological evidence  
30 found at a crime scene as a means for positively identifying, for example, a perpetrator  
of a crime. To make such an identification, PCR technology can be used to amplify  
DNA sequences taken from very small biological samples such as tissues, *e.g.*, hair or  
skin, or body fluids, *e.g.*, blood, saliva, or semen found at a crime scene. The amplified  
sequence can then be compared to a standard, thereby allowing identification of the  
35 origin of the biological sample.

The sequences of the present invention can be used to provide polynucleotide  
reagents, *e.g.*, PCR primers, targeted to specific loci in the human genome, which can  
enhance the reliability of DNA-based forensic identifications by, for example, providing

another "identification marker" (*i.e.* another DNA sequence that is unique to a particular individual). As mentioned above, actual base sequence information can be used for identification as an accurate alternative to patterns formed by restriction enzyme generated fragments. Sequences targeted to noncoding regions of SEQ ID NO:1 are particularly appropriate for this use as greater numbers of polymorphisms occur in the noncoding regions, making it easier to differentiate individuals using this technique. Examples of polynucleotide reagents include the TWIK-9 nucleotide sequences or portions thereof, *e.g.*, fragments derived from the noncoding regions of SEQ ID NO:1 having a length of at least 20 bases, preferably at least 30 bases.

10 The TWIK-9 nucleotide sequences described herein can further be used to provide polynucleotide reagents, *e.g.*, labeled or labelable probes which can be used in, for example, an *in situ* hybridization technique, to identify a specific tissue, *e.g.*, a tissue which expresses TWIK-9, *e.g.*, brain tissue. This can be very useful in cases where a forensic pathologist is presented with a tissue of unknown origin. Panels of such TWIK-9 probes can be used to identify tissue by species and/or by organ type.

In a similar fashion, these reagents, *e.g.*, TWIK-9 primers or probes can be used to screen tissue culture for contamination (*i.e.* screen for the presence of a mixture of different types of cells in a culture).

#### 20 C. Predictive Medicine:

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining TWIK-9 protein and/or nucleic acid expression as well as TWIK-9 activity, in the context of a biological sample (*e.g.*, blood, serum, cells, or tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant or unwanted TWIK-9 expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with TWIK-9 protein, nucleic acid expression, or activity. For example, mutations in a TWIK-9 gene can be assayed in a biological sample. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with TWIK-9 protein, nucleic acid expression or activity.

Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of TWIK-9 in clinical trials.

These and other agents are described in further detail in the following sections.

### 1. Diagnostic Assays

An exemplary method for detecting the presence or absence of TWIK-9 protein, polypeptide or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting TWIK-9 protein, polypeptide or nucleic acid (*e.g.*, mRNA, genomic DNA) that encodes TWIK-9 protein such that the presence of TWIK-9 protein or nucleic acid is detected in the biological sample. In another aspect, the present invention provides a method for detecting the presence of TWIK-9 activity in a biological sample by contacting the biological sample with an agent capable of detecting an indicator of TWIK-9 activity such that the presence of TWIK-9 activity is detected in the biological sample. A preferred agent for detecting TWIK-9 mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to TWIK-9 mRNA or genomic DNA. The nucleic acid probe can be, for example, a full-length TWIK-9 nucleic acid, such as the nucleic acid of SEQ ID NO:1 or 3, or the DNA insert of the plasmid deposited with ATCC as Accession Number \_\_\_\_\_, or a portion thereof, such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to TWIK-9 mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting TWIK-9 protein is an antibody capable of binding to TWIK-9 protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (*e.g.*, Fab or F(ab')<sub>2</sub>) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. That is, the detection method of the invention can be used to detect TWIK-9 mRNA, protein, or genomic DNA in a biological sample *in vitro* as well as *in vivo*. For example, *in vitro* techniques for detection of TWIK-9 mRNA include Northern hybridizations and *in situ* hybridizations. *In vitro* techniques for detection of TWIK-9 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. *In vitro* techniques for detection of TWIK-9 genomic DNA



include Southern hybridizations. Furthermore, *in vivo* techniques for detection of a TWIK-9 protein include introducing into a subject a labeled anti-TWIK-9 antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

5           The present invention also provides diagnostic assays for identifying the presence or absence of a genetic alteration characterized by at least one of (i) aberrant modification or mutation of a gene encoding a TWIK-9 protein; (ii) aberrant expression of a gene encoding a TWIK-9 protein; (iii) mis-regulation of the gene; and (iv) aberrant post-translational modification of a TWIK-9 protein, wherein a wild-type form of the  
10   gene encodes a protein with a TWIK-9 activity. "Misexpression or aberrant expression", as used herein, refers to a non-wild type pattern of gene expression, at the RNA or protein level. It includes, but is not limited to, expression at non-wild type levels (*e.g.*, over or under expression); a pattern of expression that differs from wild type in terms of the time or stage at which the gene is expressed (*e.g.*, increased or decreased expression  
15   (as compared with wild type) at a predetermined developmental period or stage); a pattern of expression that differs from wild type in terms of decreased expression (as compared with wild type) in a predetermined cell type or tissue type; a pattern of expression that differs from wild type in terms of the splicing size, amino acid sequence, post-translational modification, or biological activity of the expressed polypeptide; a  
20   pattern of expression that differs from wild type in terms of the effect of an environmental stimulus or extracellular stimulus on expression of the gene (*e.g.*, a pattern of increased or decreased expression (as compared with wild type) in the presence of an increase or decrease in the strength of the stimulus).

          In one embodiment, the biological sample contains protein molecules from the  
25   test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

          In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a  
30   compound or agent capable of detecting TWIK-9 protein, mRNA, or genomic DNA, such that the presence of TWIK-9 protein, mRNA or genomic DNA is detected in the biological sample, and comparing the presence of TWIK-9 protein, mRNA or genomic DNA in the control sample with the presence of TWIK-9 protein, mRNA or genomic DNA in the test sample.

35           The invention also encompasses kits for detecting the presence of TWIK-9 in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting TWIK-9 protein or mRNA in a biological sample; means for determining the amount of TWIK-9 in the sample; and means for comparing the amount

of TWIK-9 in the sample with a standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect TWIK-9 protein or nucleic acid.

5           2.     Prognostic Assays

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant or unwanted TWIK-9 expression or activity. As used herein, the term "aberrant" includes a TWIK-9 expression or activity which deviates from the wild type TWIK-9 expression  
10 or activity. Aberrant expression or activity includes increased or decreased expression or activity, as well as expression or activity which does not follow the wild type developmental pattern of expression or the subcellular pattern of expression. For example, aberrant TWIK-9 expression or activity is intended to include the cases in which a mutation in the TWIK-9 gene causes the TWIK-9 gene to be under-expressed or  
15 over-expressed and situations in which such mutations result in a non-functional TWIK-9 protein or a protein which does not function in a wild-type fashion, *e.g.*, a protein which does not interact with a TWIK-9 ligand or substrate, *e.g.*, a non-potassium channel subunit, or one which interacts with a non-TWIK-9 ligand or substrate. As used herein, the term "unwanted" includes an unwanted phenomenon involved in a biological  
20 response such as deregulated cell proliferation or seizure susceptibility. For example, the term unwanted includes a TWIK-9 expression or activity which is undesirable in a subject.

The assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a  
25 disorder associated with a misregulation in TWIK-9 protein activity or nucleic acid expression, such as a CNS disorder (*e.g.*, a cognitive or neurodegenerative disorder, or a pain disorder), a muscular disorder, a cardiovascular disorder, or a cellular proliferation, growth, differentiation or migration disorder. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a disorder associated  
30 with a misregulation in TWIK-9 protein activity or nucleic acid expression, such as a CNS disorder (*e.g.*, a cognitive or neurodegenerative disorder, or a pain disorder), a muscular disorder, a cardiovascular disorder, or a cellular proliferation, growth, differentiation or migration disorder. Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant or unwanted TWIK-9  
35 expression or activity in which a test sample is obtained from a subject and TWIK-9 protein or nucleic acid (*e.g.*, mRNA or genomic DNA) is detected, wherein the presence of TWIK-9 protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant or unwanted TWIK-9

expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, cerebrospinal fluid or serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine  
5 whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant or unwanted TWIK-9 expression or activity. For example, such methods can be used to determine whether a subject can be effectively treated with an agent for a CNS disorder (*e.g.*, a cognitive or  
10 neurodegenerative disorder, or a pain disorder), a muscular disorder, a cardiovascular disorder, or a cellular proliferation, growth, differentiation or migration disorder. Thus, the present invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant or unwanted TWIK-9 expression or activity in which a test sample is obtained and TWIK-9 protein  
15 or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of TWIK-9 protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant or unwanted TWIK-9 expression or activity).

The methods of the invention can also be used to detect genetic alterations in a  
20 TWIK-9 gene, thereby determining if a subject with the altered gene is at risk for a disorder characterized by misregulation in TWIK-9 protein activity or nucleic acid expression, such as a CNS disorder (*e.g.*, a cognitive or neurodegenerative disorder, or a pain disorder), a muscular disorder, a cardiovascular disorder, or a cellular proliferation, growth, differentiation or migration disorder. In preferred embodiments, the methods  
25 include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a TWIK-9-protein, or the mis-expression of the TWIK-9 gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a TWIK-9 gene; 2) an addition of  
30 one or more nucleotides to a TWIK-9 gene; 3) a substitution of one or more nucleotides of a TWIK-9 gene, 4) a chromosomal rearrangement of a TWIK-9 gene; 5) an alteration in the level of a messenger RNA transcript of a TWIK-9 gene, 6) aberrant modification of a TWIK-9 gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a TWIK-  
35 9 gene, 8) a non-wild type level of a TWIK-9-protein, 9) allelic loss of a TWIK-9 gene, and 10) inappropriate post-translational modification of a TWIK-9-protein. As described herein, there are a large number of assays known in the art which can be used

for detecting alterations in a TWIK-9 gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, *e.g.*, U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, *e.g.*, Landegran *et al.* (1988) *Science* 241:1077-1080; and Nakazawa *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:360-364), the latter of which can be particularly useful for detecting point mutations in the TWIK-9-gene (see Abravaya *et al.* (1995) *Nucleic Acids Res.* 23:675-682). This method can include the steps of collecting a sample of cells from a subject, isolating nucleic acid (*e.g.*, genomic, mRNA or both) from the cells of the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a TWIK-9 gene under conditions such that hybridization and amplification of the TWIK-9-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:1874-1878), transcriptional amplification system (Kwoh, D.Y. *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.* (1988) *Bio-Technology* 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In an alternative embodiment, mutations in a TWIK-9 gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Patent No. 5,498,531) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in TWIK-9 can be identified by hybridizing a sample and control nucleic acids, *e.g.*, DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M.T. *et al.* (1996) *Human Mutation* 7:244-255; Kozal, M.J. *et al.* (1996) *Nature Medicine* 2:753-

759). For example, genetic mutations in TWIK-9 can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M.T. *et al.* (1996) *supra*. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the  
5 sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the  
10 other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the TWIK-9 gene and detect mutations by comparing the sequence of the sample TWIK-9 with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on  
15 techniques developed by Maxam and Gilbert ((1977) *Proc. Natl. Acad. Sci. USA* 74:560) or Sanger ((1977) *Proc. Natl. Acad. Sci. USA* 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays (Naeve, C. W. (1995) *Biotechniques* 19:448), including sequencing by mass spectrometry (see, *e.g.*, PCT International Publication No.  
20 WO 94/16101; Cohen *et al.* (1996) *Adv. Chromatogr.* 36:127-162; and Griffin *et al.* (1993) *Appl. Biochem. Biotechnol.* 38:147-159).

Other methods for detecting mutations in the TWIK-9 gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers *et al.* (1985) *Science* 230:1242). In general, the  
25 art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type TWIK-9 sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample  
30 strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with S1 nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then  
35 separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton *et al.* (1988) *Proc. Natl Acad Sci USA* 85:4397; Saleeba *et al.* (1992) *Methods Enzymol.* 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in TWIK-9 cDNAs obtained from samples of cells. For example, the mutY enzyme of *E. coli* cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu *et al.* (1994) *Carcinogenesis* 15:1657-1662). According to an exemplary embodiment, a probe based on a TWIK-9 sequence, *e.g.*, a wild-type TWIK-9 sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Patent No. 5,459,039.

In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in TWIK-9 genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.* (1989) *Proc Natl. Acad. Sci USA*: 86:2766, see also Cotton (1993) *Mutat. Res.* 285:125-144; and Hayashi (1992) *Genet. Anal. Tech. Appl.* 9:73-79). Single-stranded DNA fragments of sample and control TWIK-9 nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen *et al.* (1991) *Trends Genet.* 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers *et al.* (1985) *Nature* 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) *Biophys Chem* 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions

which permit hybridization only if a perfect match is found (Saiki *et al.* (1986) *Nature* 324:163); Saiki *et al.* (1989) *Proc. Natl Acad. Sci USA* 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and  
5 hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential  
10 hybridization) (Gibbs *et al.* (1989) *Nucleic Acids Res.* 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner (1993) *Tibtech* 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.* (1992) *Mol. Cell Probes* 6:1). It is anticipated  
15 that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) *Proc. Natl. Acad. Sci USA* 88:189). In such cases, ligation will occur only if there is a perfect match at the 3' end of the 5' sequence making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

20 The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, *e.g.*, in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a TWIK-9 gene.

25 Furthermore, any cell type or tissue in which TWIK-9 is expressed may be utilized in the prognostic assays described herein.

### 3. Monitoring of Effects During Clinical Trials

Monitoring the influence of agents (*e.g.*, drugs) on the expression or activity of a  
30 TWIK-9 protein (*e.g.*, the modulation of pain signaling mechanisms, neurotransmitter release, membrane excitability and/or cell growth, proliferation, or differentiation mechanisms) can be applied not only in basic drug screening, but also in clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase TWIK-9 gene expression, protein levels, or upregulate TWIK-9  
35 activity, can be monitored in clinical trials of subjects exhibiting decreased TWIK-9 gene expression, protein levels, or downregulated TWIK-9 activity. Alternatively, the effectiveness of an agent determined by a screening assay to decrease TWIK-9 gene expression, protein levels, or downregulate TWIK-9 activity, can be monitored in

clinical trials of subjects exhibiting increased TWIK-9 gene expression, protein levels, or upregulated TWIK-9 activity. In such clinical trials, the expression or activity of a TWIK-9 gene, and preferably, other genes that have been implicated in, for example, a TWIK-9-associated disorder can be used as a “read out” or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including TWIK-9, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates TWIK-9 activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on TWIK-9-associated disorders (*e.g.*, disorders characterized by deregulated pain signaling mechanisms, K<sup>+</sup> currents, neurotransmitter release, membrane excitability, and/or cell growth, proliferation, or differentiation mechanisms), for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of TWIK-9 and other genes implicated in the TWIK-9-associated disorder, respectively. The levels of gene expression (*e.g.*, a gene expression pattern) can be quantified by northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of TWIK-9 or other genes. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of a TWIK-9 protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the TWIK-9 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the TWIK-9 protein, mRNA, or genomic DNA in the pre-administration sample with the TWIK-9 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of TWIK-9 to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of TWIK-9 to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an



embodiment, TWIK-9 expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

D. Methods of Treatment:

5 The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a TWIK-9-associated disorder, *e.g.*, a disorder associated with aberrant or unwanted TWIK-9 expression or activity, *e.g.*, a potassium channel associated disorder such as a CNS disorder, a pain disorder, a cardiovascular disorder, or a cell proliferation, growth or differentiation  
10 disorder. With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More  
15 specifically, the term refers the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's "drug response phenotype", or "drug response genotype".) Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the TWIK-9 molecules of the present invention or TWIK-9 modulators according to that individual's drug  
20 response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

25 1. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant or unwanted TWIK-9 expression or activity, by administering to the subject a TWIK-9 or an agent which modulates TWIK-9 expression or at least one TWIK-9 activity. Subjects at risk for a disease which is  
30 caused or contributed to by aberrant or unwanted TWIK-9 expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the TWIK-9 aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the  
35 type of TWIK-9 aberrancy, for example, a TWIK-9, TWIK-9 agonist or TWIK-9 antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

## 2. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating TWIK-9 expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell capable of expressing TWIK-9 with an agent that modulates one or more of the activities of TWIK-9 protein activity associated with the cell, such that TWIK-9 activity in the cell is modulated. An agent that modulates TWIK-9 protein activity can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring target molecule of a TWIK-9 protein (*e.g.*, a TWIK-9 ligand or substrate), a TWIK-9 antibody, a TWIK-9 agonist or antagonist, a peptidomimetic of a TWIK-9 agonist or antagonist, or other small molecule. In one embodiment, the agent stimulates one or more TWIK-9 activities. Examples of such stimulatory agents include active TWIK-9 protein and a nucleic acid molecule encoding TWIK-9 that has been introduced into the cell. In another embodiment, the agent inhibits one or more TWIK-9 activities. Examples of such inhibitory agents include antisense TWIK-9 nucleic acid molecules, anti-TWIK-9 antibodies, and TWIK-9 inhibitors. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of a TWIK-9 protein or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) TWIK-9 expression or activity. In another embodiment, the method involves administering a TWIK-9 protein or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted TWIK-9 expression or activity.

The term "treatment" as used herein, is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. A therapeutic agent includes, but is not limited to, small molecules, peptides, antibodies, ribozymes and antisense oligonucleotides.

Stimulation of TWIK-9 activity is desirable in situations in which TWIK-9 is abnormally downregulated and/or in which increased TWIK-9 activity is likely to have a beneficial effect. Likewise, inhibition of TWIK-9 activity is desirable in situations in

which TWIK-9 is abnormally upregulated and/or in which decreased TWIK-9 activity is likely to have a beneficial effect.

### 3. Pharmacogenomics

5       The TWIK-9 molecules of the present invention, as well as agents, or modulators which have a stimulatory or inhibitory effect on TWIK-9 activity (*e.g.*, TWIK-9 gene expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) TWIK-9-associated disorders (*e.g.*, a CNS disorder, a pain disorder, a cardiovascular disorder, or a cell proliferation, growth or differentiation disorder) associated with aberrant or unwanted TWIK-9 activity. In conjunction with such treatment, pharmacogenomics (*i.e.*, the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer a TWIK-9 molecule or TWIK-9 modulator as well as tailoring the dosage and/or therapeutic regimen of treatment with a TWIK-9 molecule or TWIK-9 modulator.

20       Pharmacogenomics deals with clinically significant hereditary variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See, for example, Eichelbaum, M. *et al.* (1996) *Clin. Exp. Pharmacol. Physiol.* 23(10-11):983-985 and Linder, M.W. *et al.* (1997) *Clin. Chem.* 43(2):254-266. In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body (altered drug action) or genetic conditions transmitted as single factors altering the way the body acts on drugs (altered drug metabolism). These pharmacogenetic conditions can occur either as rare genetic defects or as naturally-occurring polymorphisms. For example, glucose-6-phosphate methyltransferase deficiency (G6PD) is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of fava beans.

35       One pharmacogenomics approach to identifying genes that predict drug response, known as "a genome-wide association", relies primarily on a high-resolution map of the human genome consisting of already known gene-related markers (*e.g.*, a "bi-allelic" gene marker map which consists of 60,000-100,000 polymorphic or variable sites on the human genome, each of which has two variants.) Such a high-resolution genetic map can be compared to a map of the genome of each of a statistically

significant number of patients taking part in a Phase II/III drug trial to identify markers associated with a particular observed drug response or side effect. Alternatively, such a high resolution map can be generated from a combination of some ten-million known single nucleotide polymorphisms (SNPs) in the human genome. As used herein, a

5 “SNP” is a common alteration that occurs in a single nucleotide base in a stretch of DNA. For example, a SNP may occur once per every 1000 bases of DNA. A SNP may be involved in a disease process, however, the vast majority may not be disease-associated. Given a genetic map based on the occurrence of such SNPs, individuals can be grouped into genetic categories depending on a particular pattern of SNPs in their

10 individual genome. In such a manner, treatment regimens can be tailored to groups of genetically similar individuals, taking into account traits that may be common among such genetically similar individuals.

Alternatively, a method termed the “candidate gene approach” can be utilized to identify genes that predict drug response. According to this method, if a gene that

15 encodes a drug’s target is known (*e.g.*, a TWIK-9 protein of the present invention), all common variants of that gene can be fairly easily identified in the population and it can be determined if having one version of the gene versus another is associated with a particular drug response.

As an illustrative embodiment, the activity of drug metabolizing enzymes is a

20 major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (*e.g.*, N-methyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of

25 a drug. These polymorphisms are expressed in two phenotypes in the population; the extensive metabolizer (EM) and poor metabolizer (PM). The prevalence of PM is different among different populations. For example, the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM, which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite

30 frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety, PM show no therapeutic response, as demonstrated for the analgesic effect of codeine mediated by its CYP2D6-formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently, the molecular basis of

35 ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Alternatively, a method termed the “gene expression profiling”, can be utilized to identify genes that predict drug response. For example, the gene expression of an animal dosed with a drug (*e.g.*, a TWIK-9 molecule or TWIK-9 modulator of the present

invention) can give an indication whether gene pathways related to toxicity have been turned on.

Information generated from more than one of the above pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a TWIK-9 molecule or TWIK-9 modulator, such as a modulator identified by one of the exemplary screening assays described herein.

10

#### 4. Use of TWIK-9 Molecules as Surrogate Markers

The TWIK-9 molecules of the invention are also useful as markers of disorders or disease states, as markers for precursors of disease states, as markers for predisposition of disease states, as markers of drug activity, or as markers of the pharmacogenomic profile of a subject. Using the methods described herein, the presence, absence and/or quantity of the TWIK-9 molecules of the invention may be detected, and may be correlated with one or more biological states *in vivo*. For example, the TWIK-9 molecules of the invention may serve as surrogate markers for one or more disorders or disease states or for conditions leading up to disease states.

As used herein, a “surrogate marker” is an objective biochemical marker which correlates with the absence or presence of a disease or disorder, or with the progression of a disease or disorder (*e.g.*, with the presence or absence of a tumor). The presence or quantity of such markers is independent of the causation of the disease. Therefore, these markers may serve to indicate whether a particular course of treatment is effective in lessening a disease state or disorder. Surrogate markers are of particular use when the presence or extent of a disease state or disorder is difficult to assess through standard methodologies (*e.g.*, early stage tumors), or when an assessment of disease progression is desired before a potentially dangerous clinical endpoint is reached (*e.g.*, an assessment of cardiovascular disease may be made using cholesterol levels as a surrogate marker, and an analysis of HIV infection may be made using HIV RNA levels as a surrogate marker, well in advance of the undesirable clinical outcomes of myocardial infarction or fully-developed AIDS). Examples of the use of surrogate markers in the art include: Koomen *et al.* (2000) *J. Mass. Spectrom.* 35:258-264; and James (1994) *AIDS Treatment News Archive* 209.

The TWIK-9 molecules of the invention are also useful as pharmacodynamic markers. As used herein, a “pharmacodynamic marker” is an objective biochemical marker which correlates specifically with drug effects. The presence or quantity of a pharmacodynamic marker is not related to the disease state or disorder for which the

drug is being administered; therefore, the presence or quantity of the marker is indicative of the presence or activity of the drug in a subject. For example, a pharmacodynamic marker may be indicative of the concentration of the drug in a biological tissue, in that the marker is either expressed or transcribed or not expressed or transcribed in that tissue in relationship to the level of the drug. In this fashion, the distribution or uptake of the drug may be monitored by the pharmacodynamic marker. Similarly, the presence or quantity of the pharmacodynamic marker may be related to the presence or quantity of the metabolic product of a drug, such that the presence or quantity of the marker is indicative of the relative breakdown rate of the drug *in vivo*. Pharmacodynamic markers are of particular use in increasing the sensitivity of detection of drug effects, particularly when the drug is administered in low doses. Since even a small amount of a drug may be sufficient to activate multiple rounds of marker (*e.g.*, a TWIK-9 marker) transcription or expression, the amplified marker may be in a quantity which is more readily detectable than the drug itself. Also, the marker may be more easily detected due to the nature of the marker itself; for example, using the methods described herein, anti-TWIK-9 antibodies may be employed in an immune-based detection system for a TWIK-9 protein marker, or TWIK-9-specific radiolabeled probes may be used to detect a TWIK-9 mRNA marker. Furthermore, the use of a pharmacodynamic marker may offer mechanism-based prediction of risk due to drug treatment beyond the range of possible direct observations. Examples of the use of pharmacodynamic markers in the art include: Matsuda *et al.* US 6,033,862; Hattis *et al.* (1991) *Env. Health Perspect.* 90:229-238; Schentag (1999) *Am. J. Health-Syst. Pharm.* 56 Suppl. 3:S21-S24; and Nicolau (1999) *Am. J. Health-Syst. Pharm.* 56 Suppl. 3:S16-S20.

The TWIK-9 molecules of the invention are also useful as pharmacogenomic markers. As used herein, a "pharmacogenomic marker" is an objective biochemical marker which correlates with a specific clinical drug response or susceptibility in a subject (see, *e.g.*, McLeod *et al.* (1999) *Eur. J. Cancer* 35(12):1650-1652). The presence or quantity of the pharmacogenomic marker is related to the predicted response of the subject to a specific drug or class of drugs prior to administration of the drug. By assessing the presence or quantity of one or more pharmacogenomic markers in a subject, a drug therapy which is most appropriate for the subject, or which is predicted to have a greater degree of success, may be selected. For example, based on the presence or quantity of RNA, or protein (*e.g.*, TWIK-9 protein or RNA) for specific tumor markers in a subject, a drug or course of treatment may be selected that is optimized for the treatment of the specific tumor likely to be present in the subject. Similarly, the presence or absence of a specific sequence mutation in TWIK-9 DNA may correlate TWIK-9 drug response. The use of pharmacogenomic markers therefore permits the

application of the most appropriate treatment for each subject without having to administer the therapy.

#### 5. Electronic Apparatus Readable Media and Arrays

5 Electronic apparatus readable media comprising MAGK sequence information is also provided. As used herein, "MAGK sequence information" refers to any nucleotide and/or amino acid sequence information particular to the MAGK molecules of the present invention, including but not limited to full-length nucleotide and/or amino acid sequences, partial nucleotide and/or amino acid sequences, polymorphic sequences  
10 including single nucleotide polymorphisms (SNPs), epitope sequences, and the like. Moreover, information "related to" said MAGK sequence information includes detection of the presence or absence of a sequence (*e.g.*, detection of expression of a sequence, fragment, polymorphism, etc.), determination of the level of a sequence (*e.g.*, detection of a level of expression, for example, a quantitative detection), detection of a reactivity to  
15 a sequence (*e.g.*, detection of protein expression and/or levels, for example, using a sequence-specific antibody), and the like. As used herein, "electronic apparatus readable media" refers to any suitable medium for storing, holding or containing data or information that can be read and accessed directly by an electronic apparatus. Such media can include, but are not limited to: magnetic storage media, such as floppy discs,  
20 hard disc storage medium, and magnetic tape; optical storage media such as compact disc; electronic storage media such as RAM, ROM, EPROM, EEPROM and the like; general hard disks and hybrids of these categories such as magnetic/optical storage media. The medium is adapted or configured for having recorded thereon MAGK sequence information of the present invention.

25 As used herein, the term "electronic apparatus" is intended to include any suitable computing or processing apparatus or other device configured or adapted for storing data or information. Examples of electronic apparatus suitable for use with the present invention include stand-alone computing apparatus; networks, including a local area network (LAN), a wide area network (WAN) Internet, Intranet, and Extranet;  
30 electronic appliances such as a personal digital assistants (PDAs), cellular phone, pager and the like; and local and distributed processing systems.

As used herein, "recorded" refers to a process for storing or encoding information on the electronic apparatus readable medium. Those skilled in the art can readily adopt any of the presently known methods for recording information on known  
35 media to generate manufactures comprising the MAGK sequence information.

A variety of software programs and formats can be used to store the sequence information on the electronic apparatus readable medium. For example, the sequence information can be represented in a word processing text file, formatted in

commercially-available software such as WordPerfect and MicroSoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like, as well as in other forms. Any number of data processor structuring formats (*e.g.*, text file or database) may be employed in order to obtain or  
5 create a medium having recorded thereon the MAGK sequence information.

By providing MAGK sequence information in readable form, one can routinely access the sequence information for a variety of purposes. For example, one skilled in the art can use the sequence information in readable form to compare a target sequence or target structural motif with the sequence information stored within the data storage  
10 means. Search means are used to identify fragments or regions of the sequences of the invention which match a particular target sequence or target motif.

The present invention therefore provides a medium for holding instructions for performing a method for determining whether a subject has a MAGK- associated disease or disorder or a pre-disposition to a MAGK-associated disease or disorder, wherein the  
15 method comprises the steps of determining MAGK sequence information associated with the subject and based on the MAGK sequence information, determining whether the subject has a MAGK -associated disease or disorder or a pre-disposition to a MAGK-associated disease or disorder and/or recommending a particular treatment for the disease, disorder or pre-disease condition.

20 The present invention further provides in an electronic system and/or in a network, a method for determining whether a subject has a MAGK-associated disease or disorder or a pre-disposition to a disease associated with a MAGK wherein the method comprises the steps of determining MAGK sequence information associated with the subject, and based on the MAGK sequence information, determining whether the subject  
25 has a MAGK -associated disease or disorder or a pre-disposition to a MAGK-associated disease or disorder, and/or recommending a particular treatment for the disease, disorder or pre-disease condition. The method may further comprise the step of receiving phenotypic information associated with the subject and/or acquiring from a network phenotypic information associated with the subject.

30 The present invention also provides in a network, a method for determining whether a subject has a MAGK-associated disease or disorder or a pre-disposition to a MAGK associated disease or disorder associated with MAGK, said method comprising the steps of receiving MAGK sequence information from the subject and/or information related thereto, receiving phenotypic information associated with the subject, acquiring  
35 information from the network corresponding to MAGK and/or a MAGK-associated disease or disorder, and based on one or more of the phenotypic information, the MAGK information (*e.g.*, sequence information and/or information related thereto), and the acquired information, determining whether the subject has a MAGK-associated disease



or disorder or a pre-disposition to a MAGK-associated disease or disorder (*e.g.*, a cellular growth or proliferation disease or disorder, for example, cancer). The method may further comprise the step of recommending a particular treatment for the disease, disorder or pre-disease condition.

5           The present invention also provides a business method for determining whether a subject has a MAGK-associated disease or disorder or a pre-disposition to a MAGK-associated disease or disorder, said method comprising the steps of receiving information related to MAGK (*e.g.*, sequence information and/or information related thereto), receiving phenotypic information associated with the subject, acquiring  
10 information from the network related to MAGK and/or related to a MAGK-associated disease or disorder, and based on one or more of the phenotypic information, the MAGK information, and the acquired information, determining whether the subject has a MAGK-associated disease or disorder or a pre-disposition to a MAGK-associated disease or disorder. The method may further comprise the step of recommending a  
15 particular treatment for the disease, disorder or pre-disease condition.

          The invention also includes an array comprising a MAGK sequence of the present invention. The array can be used to assay expression of one or more genes in the array. In one embodiment, the array can be used to assay gene expression in a tissue to ascertain tissue specificity of genes in the array. In this manner, up to about 7600 genes  
20 can be simultaneously assayed for expression, one of which can be MAGK. This allows a profile to be developed showing a battery of genes specifically expressed in one or more tissues.

          In addition to such qualitative determination, the invention allows the quantitation of gene expression. Thus, not only tissue specificity, but also the level of  
25 expression of a battery of genes in the tissue is ascertainable. Thus, genes can be grouped on the basis of their tissue expression *per se* and level of expression in that tissue. This is useful, for example, in ascertaining the relationship of gene expression between or among tissues. Thus, one tissue can be perturbed and the effect on gene expression in a second tissue can be determined. In this context, the effect of one cell  
30 type on another cell type in response to a biological stimulus can be determined. Such a determination is useful, for example, to know the effect of cell-cell interaction at the level of gene expression. If an agent is administered therapeutically to treat one cell type but has an undesirable effect on another cell type, the invention provides an assay to determine the molecular basis of the undesirable effect and thus provides the  
35 opportunity to co-administer a counteracting agent or otherwise treat the undesired effect. Similarly, even within a single cell type, undesirable biological effects can be determined at the molecular level. Thus, the effects of an agent on expression of other than the target gene can be ascertained and counteracted.

In another embodiment, the array can be used to monitor the time course of expression of one or more genes in the array. This can occur in various biological contexts, as disclosed herein, for example development of a MAGK-associated disease or disorder, progression of MAGK-associated disease or disorder, and processes, such as cellular transformation associated with the MAGK-associated disease or disorder.

The array is also useful for ascertaining the effect of the expression of a gene on the expression of other genes in the same cell or in different cells (*e.g.*, ascertaining the effect of MAGK expression on the expression of other genes). This provides, for example, for a selection of alternate molecular targets for therapeutic intervention if the ultimate or downstream target cannot be regulated.

The array is also useful for ascertaining differential expression patterns of one or more genes in normal and abnormal cells. This provides a battery of genes (*e.g.*, including MAGK) that could serve as a molecular target for diagnosis or therapeutic intervention.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the figures and the appendices, are incorporated herein by reference.

## EXAMPLES

### EXAMPLE 1: IDENTIFICATION AND CHARACTERIZATION OF HUMAN TWIK-9 cDNA

In this example, the identification and characterization of the gene encoding human TWIK-9 (clone Fbh56115) is described.

#### Isolation of the human TWIK-9 cDNA

The invention is based, at least in part, on the discovery of a human gene encoding a novel member of the TWIK-like family of potassium channels. The entire sequence of human clone Fbh56115 was determined and found to contain an open reading frame termed human "TWIK-9".

The nucleotide sequence encoding the human TWIK-9 is shown in Figure 1 and is set forth as SEQ ID NO:1. The protein encoded by this nucleic acid comprises about 374 amino acids and has the amino acid sequence shown in Figure 1 and set forth as SEQ ID NO:2. The coding region (open reading frame) of SEQ ID NO:1 is set forth as SEQ ID NO:3. Clone Fbh56115, comprising the coding region of human TWIK-9, was

deposited with the American Type Culture Collection (ATCC®), 10801 University Boulevard, Manassas, VA 20110-2209, on \_\_, and assigned Accession No. \_\_\_\_\_.

#### Analysis of the human TWIK-9 Molecules

5           The amino acid sequence set forth as SEQ ID NO:2 was searched against the Washington University HMM database (Figure 3). This search resulted in the identification of the protein of SEQ ID NO:2 as a "TWIK channel" family member, e.g., as having a score of 587.6 when aligned to the TWIK family HMM having PFAM Accession No. PF02034.

10           Analysis of the amino acid sequence of human TWIK-9 was also performed using MEMSAT. This analysis resulted in the identification of potential transmembrane domains in the amino acid sequence of human TWIK-9 at residues 9-26, 80-96, 108-125, 159-178, 190-208 and 219-243 of SEQ ID NO:2. Further analysis of the TWIK-9 amino acid sequence resulted in a determination that residues 80-96 and 190-208 of  
15   SEQ ID NO:2, in fact, correspond to P-loops or pores, as defined herein. Yet further analysis of the TWIK-9 amino acid sequence resulted in a determination that residues 9-26, 108-125, 159-178 and 219-243 correspond to transmembrane domain 1, transmembrane domain 2, transmembrane domain 3 and transmembrane domain 4, respectively. Moreover, amino acid residues 346-362 are predicted not to correspond to  
20   a transmembrane domain due, at least in part, to the low score of 0.2

          The amino acid sequence of human TWIK-9 was also analyzed using the program PSORT (<http://www.psорт.nibb.ac.jp>) to predict potential localization of the protein within the cell. This program assesses the presence of different targeting and localization amino acid sequences within the query sequence. The results of the  
25   analyses show that human TWIK-9 could be localized to the endoplasmic reticulum, if found intracellularly.

          A SignalP signal peptide prediction program also recognized the hydrophobic nature of transmembrane domain 1 as a possible signal sequence, e.g., at about amino acids 1-24 of SEQ ID NO:2, although signal and structural analysis as described above  
30   indicates more strongly that residues 9-26 are a transmembrane domain.

          Searches of the amino acid sequence of human TWIK-9 were further performed against the Prosite database, and resulted in the identification of several possible phosphorylation sites within the amino acid sequence of human TWIK-9 (SEQ ID NO:2). Protein kinase C phosphorylation sites were identified at residues 319-321, 331-  
35   333 and 341-343; a cAMP and cGMP dependent protein kinase phosphorylation site was identified at residues 370-373; and casein kinase II phosphorylation sites were identified at residues 31-34, 55-58, 127-130, 179-182, 251-254 and 360-363 of human TWIK-9. The search also identified the presence of a N-glycosylation site motif at

amino acid residues 53-56; and N-myristoylation site motifs at amino acid residues 102-107, 117-122, 148-153, 236-241 and 266-271 of human TWIK-9.

To further identify potential structural and/or functional properties in a protein of interest, the amino acid sequence of the protein is searched against a database of annotated protein domains (*e.g.*, the ProDom database) using the default parameters (available at <http://www.toulouse.inra.fr/prodom.html>). A search of the amino acid sequence of human TWIK-9 (SEQ ID NO:2) was performed against the ProDom database. This search resulted in the local alignment of the human TWIK-9 protein with various potassium channel proteins. Specifically, amino acid residues 1-77, 75-212, 78-246, 78-247, 247-327, 288-374 and 328-374 of SEQ ID NO:2 have significant identity to various potassium channel proteins, *e.g.*, various TWIK-related, acid-sensitive, potassium channels and/or other potassium channel subfamily members.

#### Tissue Distribution of TWIK-9 mRNA

TWIK-9 expression was also determined by PCR analysis of cDNA libraries from various tissues and cell lines. Detecting expression by a library array procedure entailed preparing a PCR mixture including Taq Polymerase, dNTPs, and PCR buffer, and adding a vector primer, a primer internal to the gene of interest, and an aliquot of a library in which expression was to be tested. This procedure was performed with many libraries at a time in a 96 well PCR tray, with 80 or more wells containing libraries and a control well in which the above primers were combined with the clone of interest itself. The control well served as an indicator of the fragment size to be expected in the library wells, in the event the clone of interest was expressed within. Amplification was performed in a PCR machine, employing standard PCR conditions for denaturing, annealing, and elongation, and the resultant mixture was mixed with an appropriate loading dye and run on an ethidium bromide-stained agarose gel. The gel was later viewed with UV light after the DNA loaded within its lanes had time to migrate into the gels. Lanes in which a band corresponding with the control band was visible indicated the libraries in which the clone of interest was expressed.

TWIK-9 was expressed in mouse fetal brain, fetal thymus, cerebellum, dorsal root ganglia, testes, and mammary gland, as well as in Th2 induced T cells and WI-38 cells released from serum starvation.

#### **EXAMPLE 2: EXPRESSION OF RECOMBINANT TWIK-9 PROTEIN IN BACTERIAL CELLS**

In this example, human TWIK-9 is expressed as a recombinant glutathione-S-transferase (GST) fusion polypeptide in *E. coli* and the fusion polypeptide is isolated and characterized. Specifically, human TWIK-9 is fused to GST and this fusion polypeptide is expressed in *E. coli*, e.g., strain PEB199. Expression of the GST-TWIK-9 fusion protein in PEB199 is induced with IPTG. The recombinant fusion polypeptide is purified from crude bacterial lysates of the induced PEB199 strain by affinity chromatography on glutathione beads. Using polyacrylamide gel electrophoretic analysis of the polypeptide purified from the bacterial lysates, the molecular weight of the resultant fusion polypeptide is determined.

### EXAMPLE 3: EXPRESSION OF RECOMBINANT TWIK-9 PROTEIN IN COS CELLS

To express the TWIK-9 gene in COS cells, the pcDNA/Amp vector by Invitrogen Corporation (San Diego, CA) is used. This vector contains an SV40 origin of replication, an ampicillin resistance gene, an *E. coli* replication origin, a CMV promoter followed by a polylinker region, and an SV40 intron and polyadenylation site. A DNA fragment encoding the entire TWIK-9 protein and an HA tag (Wilson *et al.* (1984) *Cell* 37:767) or a FLAG tag fused in-frame to its 3' end of the fragment is cloned into the polylinker region of the vector, thereby placing the expression of the recombinant protein under the control of the CMV promoter.

To construct the plasmid, the TWIK-9 DNA sequence is amplified by PCR using two primers. The 5' primer contains the restriction site of interest followed by approximately twenty nucleotides of the TWIK-9 coding sequence starting from the initiation codon; the 3' end sequence contains complementary sequences to the other restriction site of interest, a translation stop codon, the HA tag or FLAG tag and the last 20 nucleotides of the TWIK-9 coding sequence. The PCR amplified fragment and the pcDNA/Amp vector are digested with the appropriate restriction enzymes and the vector is dephosphorylated using the CIAP enzyme (New England Biolabs, Beverly, MA). Preferably the two restriction sites chosen are different so that the TWIK-9 gene is inserted in the correct orientation. The ligation mixture is transformed into *E. coli* cells (strains HB101, DH5 $\alpha$ , SURE, available from Stratagene Cloning Systems, La Jolla, CA, can be used), the transformed culture is plated on ampicillin media plates, and resistant colonies are selected. Plasmid DNA is isolated from transformants and examined by restriction analysis for the presence of the correct fragment.

COS cells are subsequently transfected with the TWIK-9-pcDNA/Amp plasmid DNA using the calcium phosphate or calcium chloride co-precipitation methods, DEAE-dextran-mediated transfection, lipofection, or electroporation. Other suitable methods

- for transfecting host cells can be found in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2<sup>nd</sup>, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The expression of the TWIK-9 polypeptide is detected by radiolabelling (<sup>35</sup>S-methionine or <sup>35</sup>S-
- 5 cysteine available from NEN, Boston, MA, can be used) and immunoprecipitation (Harlow, E. and Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988) using an HA specific monoclonal antibody. Briefly, the cells are labeled for 8 hours with <sup>35</sup>S-methionine (or <sup>35</sup>S-
- 10 cysteine). The culture media are then collected and the cells are lysed using detergents (RIPA buffer, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 50 mM Tris, pH 7.5). Both the cell lysate and the culture media are precipitated with an HA specific monoclonal antibody. Precipitated polypeptides are then analyzed by SDS-PAGE.

Alternatively, DNA containing the TWIK-9 coding sequence is cloned directly into the polylinker of the pCDNA/Amp vector using the appropriate restriction sites.

- 15 The resulting plasmid is transfected into COS cells in the manner described above, and the expression of the TWIK-9 polypeptide is detected by radiolabelling and immunoprecipitation using a TWIK-9 specific monoclonal antibody.

## 20 Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. An isolated nucleic acid molecule selected from the group consisting of:
  - (a) a nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO:1; and
  - 5 (b) a nucleic acid molecule comprising the nucleotide sequence set forth in SEQ ID NO:3.
2. An isolated nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2.
- 10 3. An isolated nucleic acid molecule comprising the nucleotide sequence contained in the plasmid deposited with ATCC® as Accession Number \_\_\_\_\_.
4. An isolated nucleic acid molecule which encodes a naturally-occurring  
15 allelic variant of a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2.
5. An isolated nucleic acid molecule selected from the group consisting of:
  - (a) a nucleic acid molecule comprising a nucleotide sequence which is at  
20 least 60% identical to the nucleotide sequence of SEQ ID NO:1 or 3, or a complement thereof;
  - (b) a nucleic acid molecule comprising a fragment of at least 30 nucleotides of a nucleic acid comprising the nucleotide sequence of SEQ ID NO:1 or 3, or a complement thereof;
  - 25 (c) a nucleic acid molecule which encodes a polypeptide comprising an amino acid sequence at least about 60% identical to the amino acid sequence of SEQ ID NO:2; and
  - (d) a nucleic acid molecule which encodes a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises  
30 at least 10 contiguous amino acid residues of the amino acid sequence of SEQ ID NO:2.
6. An isolated nucleic acid molecule which hybridizes to a complement of the nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5 under stringent conditions.
- 35 7. An isolated nucleic acid molecule comprising a nucleotide sequence which is complementary to the nucleotide sequence of the nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5.

8. An isolated nucleic acid molecule comprising the nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5, and a nucleotide sequence encoding a heterologous polypeptide.
- 5 9. A vector comprising the nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5.
- 10 10. The vector of claim 9, which is an expression vector.
- 11 11. A host cell transfected with the expression vector of claim 10.
12. A method of producing a polypeptide comprising culturing the host cell of claim 11 in an appropriate culture medium to, thereby, produce the polypeptide.
- 15 13. An isolated polypeptide selected from the group consisting of:
- a) a fragment of a polypeptide comprising the amino acid sequence of SEQ ID NO:2, wherein the fragment comprises at least 10 contiguous amino acids of SEQ ID NO:2;
- b) a naturally occurring allelic variant of a polypeptide comprising  
20 the amino acid sequence of SEQ ID NO:2, wherein the polypeptide is encoded by a nucleic acid molecule which hybridizes to complement of a nucleic acid molecule consisting of SEQ ID NO:1 or 3 under stringent conditions;
- c) a polypeptide which is encoded by a nucleic acid molecule comprising a nucleotide sequence which is at least 60 % identical to a nucleic acid  
25 comprising the nucleotide sequence of SEQ ID NO:1 or 3; and
- d) a polypeptide comprising an amino acid sequence which is at least 60% identical to the amino acid sequence of SEQ ID NO:2.
14. The isolated polypeptide of claim 13 comprising the amino acid sequence  
30 of SEQ ID NO:2.
15. The polypeptide of claim 13, further comprising heterologous amino acid sequences.
- 35 16. An antibody which selectively binds to a polypeptide of claim 13.
17. A method for detecting the presence of a polypeptide of claim 13 in a sample comprising:



- a) contacting the sample with a compound which selectively binds to the polypeptide; and
- b) determining whether the compound binds to the polypeptide in the sample to thereby detect the presence of a polypeptide of claim 13 in the sample.

5

18. The method of claim 17, wherein the compound which binds to the polypeptide is an antibody.

10 19. A kit comprising a compound which selectively binds to a polypeptide of claim 13 and instructions for use.

20. A method for detecting the presence of a nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5 in a sample comprising:

- a) contacting the sample with a nucleic acid probe or primer which  
15 selectively hybridizes to the nucleic acid molecule; and
- b) determining whether the nucleic acid probe or primer binds to a nucleic acid molecule in the sample to thereby detect the presence of a nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5 in the sample.

20 21. The method of claim 20, wherein the sample comprises mRNA molecules and is contacted with a nucleic acid probe.

22. A kit comprising a compound which selectively hybridizes to a nucleic acid molecule of any one of claims 1, 2, 3, 4, or 5 and instructions for use.

25

23. A method for identifying a compound which binds to a polypeptide of claim 13 comprising:

- a) contacting the polypeptide, or a cell expressing the polypeptide with a test compound; and
- 30 b) determining whether the polypeptide binds to the test compound.

24. The method of claim 23, wherein the binding of the test compound to the polypeptide is detected by a method selected from the group consisting of:

- a) detection of binding by direct detection of test  
35 compound/polypeptide binding;
- b) detection of binding using a competition binding assay; and
- c) detection of binding using an assay for TWIK-9 activity.

25. A method for modulating the activity of a polypeptide of claim 13 comprising contacting the polypeptide or a cell expressing the polypeptide with a compound which binds to the polypeptide in a sufficient concentration to modulate the activity of the polypeptide.

5

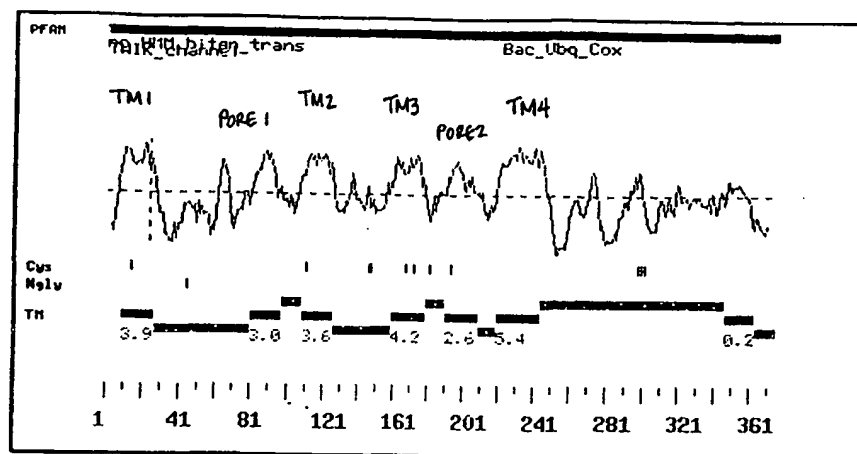
26. A method for identifying a compound which modulates the activity of a polypeptide of claim 13 comprising:

- a) contacting a polypeptide of claim 13 with a test compound; and
  - b) determining the effect of the test compound on the activity of the
- 10 polypeptide to thereby identify a compound which modulates the activity of the polypeptide.

Input file Fbh56115FL.seq; Output File Fbh56115FL.tra  
Sequence length 1262

M K R Q N V R T L S L I V C T F	16
CCTTGCTGGCGGCC ATG AAG AGG CAG AAC GTG CGG ACT CTG TCC CTC ATC GTC TGC ACC TTC	48
T Y L L V G A A V F D A L E S D H E M R	36
ACC TAC CTG CTG GTG GGC GCC GCC GTG TTC GAC GCC CTC GAG TCG GAC CAC GAG ATG CGC	108
E E E K L K A E E I R I K G K Y N I S S	56
GAG GAG GAG AAA CTC AAA GCC GAG GAG ATC CGG ATC AAG GGG AAG TAC AAC ATC AGC AGC	168
E D Y R Q L E L V I L Q S E P H R A G V	76
GAG GAC TAC CGS CAG CTG GAG CTG GTG ATC CTG CAG TCG GAA CCG CAC CGC GCC GGC GTC	228
Q W K F A G S F Y F A I T V I T T I G Y	96
CAG TGG AAA TTC GCC GGC TCC TTC TAC TTT GCG ATC ACG GTC ATC ACC ACC ATA GGT TAT	288
G H A A P G T D A G K A F C M F Y A V L	116
GGG CAC GCT GCA CCT GGC ACC GAT GCG GGC AAG GCC TTC TGC ATG TTC TAC GCC GTG CTG	348
G I P L T L V M F Q S L G E R M N T F V	136
GGC ATC CCG CTG ACA CTG GTC ATG TTT CAG AGC CTG GGC GAG CGC ATG AAC ACC TTC GTG	408
R Y L L K R I K K C C G M R N T D V S M	156
CGC TAC CTG CTG AAG CGC ATT AAG AAG TGC TGT GGC ATG CGC AAC ACT GAC GTG TCT ATG	468
E N M V T V G F F S C M G T L C I G A A	176
GAG AAC ATG GTG ACT GTG GGC TTC TTC TCC TGC ATG GGG ACG CTG TGC ATC GGG GCG GCC	528
A F S Q C E E W S F F H A Y Y Y C F I T	196
GCC TTC TCC CAG TGT GAG GAG TGG AGC TTC TTC CAC GCC TAC TAC TAC TGC TTC ATC ACG	588
L T T I G F G D Y V A L Q T K G A L Q K	216
TTG ACT ACC ATT GGG TTC GGG GAC TAC GTG GCC CTG CAG ACC AAG GGC GCC CTG CAG AAG	648
K P L Y V A F S F M Y I L V G L T V I G	236
AAG CCG CTC TAC GTG GCC TTT AGC TTT ATG TAT ATC CTG GTG GGG CTG ACG GTC ATC GGG	708
A F L N L V V L R F L T M N S E D E R R	256
GCC TTC CTC AAC CTG GTC GTC CTC AGG TTC TTG ACC ATG AAC AGT GAG GAT GAG CGG CGG	768
D A E E R A S L A G N R N S M V I H I P	276
GAT GCT GAA GAG AGG GCA TCC CTC GCC GGA AAC CGC AAC AGC ATG GTC ATT CAC ATC CCT	828
E E P R P S R P R Y K A D V P D L Q S V	296
GAG GAG CCG CGG CCC AGC CGG CCC AGG TAC AAG GCG GAC GTC CCG GAC CTG CAG TCT GTG	888
C S C T C Y R S Q D Y G G R S V A P Q N	316
TGC TCC TGC ACC TGC TAC CGC TCG CAG GAC TAT GGC GGC CGC TCG GTG GCA CCG CAG AAC	948
S F S A K L A P H Y F H S I S Y K I E E	336
TCC TTC AGC GCC AAG CTT GCC CCC CAC TAC TTC CAC TCC ATC TCT TAC AAG ATC GAG GAG	1008
I S P S T L K N S L F P S P I S S I S P	356
ATC TCA CCA AGC ACA TTA AAA AAC AGC CTC TTC CCA TCG CCT ATT AGC TCC ATC TCT CCT	1068
G L H S F T D H Q R L M K R R K S V *	375
GGG TTA CAC AGC TTT ACC GAC CAC CAG AGG CTG ATG AAA CGC CGG AAG TCC GTT TAG	1125
GGGAACATAACTGCACATTCAAGAGAGGCGTCCGTGGATGCTGGGTCTCACTGCCAAAGCCGAACACGGCTTCGGGATTT	
CTTGCCCTTCTCAAGTGGACCTCTTGCTGTGCTGGCGGAATGCC	

FIGURE 1



>Fbh56115  
 MKRQNVRTLSLIVCTFTYLLVGAADFDALESDHEMREEERLKAEIIRIKGKYNISSELYR  
 QLELVILQSEPHRAGVQWKFAGSPYFAITVITIGYGHAAFGTDAGKAFPCMFYAVLGIPL  
 TLVMPQSLGERMNTFVRYLLKRIKKCCGHRNTDVSMENMVTGPFSCMTLCIGAAAFSQ  
 CEEMSPFHAYYCFITLTTIGFGDYVALQTKGALQKKPLYVAFSFMYLVLGLTVIGAFIN  
 LVVLRLTHNSEDERDAEERASLAGNRNSMVIHIEEPRPSRPRYKADVPDLQSVCSCT  
 CYRSQDYGGRSVAPQNSFSAKLAPHYFHSISYKIEEISPSTLKNLFPSPISISPGLHS  
 FTDHQRIMKRRKSV

**FIGURE 2**

Query: Fb456115

**Parsed for domains:**

Alignments of top-scoring domains:

**Bac\_Ubg\_Cox:** domain 1 of 1, from 221 to 256; score 2.8, E = 2.4

Fbh56115 221 VAPSFMYILVGLTVIGAFNLVVLRLTNSEDERR 256

**TRIK\_channel: domain 1 of 1, from 1 to 374: score 587.6, E = 7.7e-173**

[illegible]

### FIGURE 3

## SEQUENCE LISTING

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<120> 56115, A NOVEL HUMAN TWIK POTASSIUM CHANNEL AND USES THEREFOR

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gtc tgc acc ttc acc tac ctg ctg gtg ggc gcc gcc gtg ttc gac gcc      98
Val Cys Thr Phe Thr Tyr Leu Leu Val Gly Ala Ala Val Phe Asp Ala
      15              20              25

ctc gag tcg gac cac gag atg cgc gag gag gag aaa ctc aaa gcc gag    146
Leu Glu Ser Asp His Glu Met Arg Glu Glu Glu Lys Leu Lys Ala Glu
      30              35              40

gag atc cgg atc aag ggg aag tac aac atc agc agc gag gac tac cgg    194
Glu Ile Arg Ile Lys Gly Lys Tyr Asn Ile Ser Ser Glu Asp Tyr Arg
      45              50              55              60

cag ctg gag ctg gtg atc ctg cag tcg gaa ccg cac cgc gcc gcc gtc    242
Gln Leu Glu Leu Val Ile Leu Gln Ser Glu Pro His Arg Ala Gly Val
              65              70              75

cag tgg aaa ttc gcc gcc tcc ttc tac ttt gcg atc acg gtc atc acc    290
Gln Trp Lys Phe Ala Gly Ser Phe Tyr Phe Ala Ile Thr Val Ile Thr
              80              85              90

acc ata ggt tat ggg cac gct gca cct ggc acc gat gcg gcc aag gcc    338
Thr Ile Gly Tyr Gly His Ala Ala Pro Gly Thr Asp Ala Gly Lys Ala
              95              100              105

ttc tgc atg ttc tac gcc gtg ctg ggc atc ccg ctg aca ctg gtc atg    386
Phe Cys Met Phe Tyr Ala Val Leu Gly Ile Pro Leu Thr Leu Val Met
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Lys Arg Ile Lys Lys Cys Cys Gly Met Arg Asn Thr Asp Val Ser Met	
145 150 155	
gag aac atg gtg act gtg ggc ttc ttc tcc tgc atg ggg acg ctg tgc	530
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Ile Gly Ala Ala Ala Phe Ser Gln Cys Glu Glu Trp Ser Phe Phe His	
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gat gag cgg cgg gat gct gaa gag agg gca tcc ctc gcc gga aac cgc	818
Asp Glu Arg Arg Asp Ala Glu Glu Arg Ala Ser Leu Ala Gly Asn Arg	
255 260 265	
aac agc atg gtc att cac atc cct gag gag ccg cgg ccc agc cgg ccc	866
Asn Ser Met Val Ile His Ile Pro Glu Glu Pro Arg Pro Ser Arg Pro	
270 275 280	
agg tac aag gcg gac gtc ccg gac ctg cag tct gtg tgc tcc tgc acc	914
Arg Tyr Lys Ala Asp Val Pro Asp Leu Gln Ser Val Cys Ser Cys Thr	
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tgc tac cgc tcg cag gac tat ggc ggc cgc tcg gtg gca ccg cag aac	962
Cys Tyr Arg Ser Gln Asp Tyr Gly Gly Arg Ser Val Ala Pro Gln Asn	
305 310 315	
tcc ttc agc gcc aag ctt gcc ccc cac tac ttc cac tcc atc tct tac	1010
Ser Phe Ser Ala Lys Leu Ala Pro His Tyr Phe His Ser Ile Ser Tyr	
320 325 330	
aag atc gag gag atc tca cca agc aca tta aaa aac agc ctc ttc cca	1058
Lys Ile Glu Glu Ile Ser Pro Ser Thr Leu Lys Asn Ser Leu Phe Pro	
335 340 345	
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<213> Homo sapiens

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 20 25 30

His Glu Met Arg Glu Glu Glu Lys Leu Lys Ala Glu Glu Ile Arg Ile  
 35 40 45

Lys Gly Lys Tyr Asn Ile Ser Ser Glu Asp Tyr Arg Gln Leu Glu Leu  
 50 55 60

Val Ile Leu Gln Ser Glu Pro His Arg Ala Gly Val Gln Trp Lys Phe  
 65 70 75 80

Ala Gly Ser Phe Tyr Phe Ala Ile Thr Val Ile Thr Thr Ile Gly Tyr  
 85 90 95

Gly His Ala Ala Pro Gly Thr Asp Ala Gly Lys Ala Phe Cys Met Phe  
 100 105 110

Tyr Ala Val Leu Gly Ile Pro Leu Thr Leu Val Met Phe Gln Ser Leu  
 115 120 125

Gly Glu Arg Met Asn Thr Phe Val Arg Tyr Leu Leu Lys Arg Ile Lys  
 130 135 140

Lys Cys Cys Gly Met Arg Asn Thr Asp Val Ser Met Glu Asn Met Val  
 145 150 155 160

Thr Val Gly Phe Phe Ser Cys Met Gly Thr Leu Cys Ile Gly Ala Ala  
 165 170 175

Ala Phe Ser Gln Cys Glu Glu Trp Ser Phe Phe His Ala Tyr Tyr Tyr  
 180 185 190

Cys Phe Ile Thr Leu Thr Thr Ile Gly Phe Gly Asp Tyr Val Ala Leu  
 195 200 205

Gln Thr Lys Gly Ala Leu Gln Lys Lys Pro Leu Tyr Val Ala Phe Ser  
 210 215 220

Phe Met Tyr Ile Leu Val Gly Leu Thr Val Ile Gly Ala Phe Leu Asn  
 225 230 235 240



Leu	Val	Val	Leu	Arg	Phe	Leu	Thr	Met	Asn	Ser	Glu	Asp	Glu	Arg	Arg	
				245					250					255		
Asp	Ala	Glu	Glu	Arg	Ala	Ser	Leu	Ala	Gly	Asn	Arg	Asn	Ser	Met	Val	
				260					265					270		
Ile	His	Ile	Pro	Glu	Glu	Pro	Arg	Pro	Ser	Arg	Pro	Arg	Tyr	Lys	Ala	
				275					280					285		
Asp	Val	Pro	Asp	Leu	Gln	Ser	Val	Cys	Ser	Cys	Thr	Cys	Tyr	Arg	Ser	
				290					295					300		
Gln	Asp	Tyr	Gly	Gly	Arg	Ser	Val	Ala	Pro	Gln	Asn	Ser	Phe	Ser	Ala	
				305					310					315		
Lys	Leu	Ala	Pro	His	Tyr	Phe	His	Ser	Ile	Ser	Tyr	Lys	Ile	Glu	Glu	
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1		5			10					15							
acc	tac	ctg	ctg	gtg	ggc	gcc	gcc	gtg	ttc	gac	gcc	ctc	gag	tcg	gac	96	
Thr	Tyr	Leu	Leu	Val	Gly	Ala	Ala	Val	Phe	Asp	Ala	Leu	Glu	Ser	Asp		
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Ala	Gly	Ser	Phe	Tyr	Phe	Ala	Ile	Thr	Val	Ile	Thr	Thr	Ile	Gly	Tyr		
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370	

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33/53, C12Q 1/68

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patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
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(54) Title: 56115, A NOVEL HUMAN TWIK POTASSIUM CHANNEL AND USES THEREFOR

(57) Abstract: The invention provides isolated nucleic acid molecules, designated TWIK-9 nucleic acid molecules, which encode novel TWIK-related potassium channel subunit molecules. The innovation also provides antisense nucleic acid molecules, recombinant expression vectors containing TWIK-9 nucleic acid molecules, host cells into which the expression vectors have been introduced, and nonhuman transgenic animals in which a TWIK-9 gene has been introduced or disrupted. The invention still further provides isolated TWIK-9 proteins, fusion proteins, antigenic peptides and anti-TWIK-9 antibodies. Diagnostic methods utilizing compositions of the invention are also provided.

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 01/30048

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/705 C12N5/10 C07K16/18 G01N33/50  
G01N33/53 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, EMBL, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RAJAN ET AL: "TASK-3, a Novel Tandem Pore Domain Acid-Sensitive K1 Channel. An Extracellular Histidine as pH Sensor" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, 'Online! vol. 275, no. 22, 2 June 2000 (2000-06-02), pages 16650-16657, XP002146308 ISSN: 0021-9258 AF21289 with 99.8% identity over 1125 nt (whole length cDNA) and Q9NPC2 with 100% identity over the whole length polypeptide. the whole document	1,2,4-26
X	-& DATABASE EMBL 'Online! 14 April 2000 (2000-04-14) RAJAN ET AL.: "TASK-3" retrieved from EBI -/--	1,2,4-26

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the International filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the International filing date but later than the priority date claimed

- \*T\* later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the International search

15 November 2002

Date of mailing of the International search report

03/12/2002

Name and mailing address of the ISA

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NL - 2280 HV Rijswijk  
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Authorized officer

Friedrich, C

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/30048

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	<p>Database accession no. AF212829 XP002220522 100% identity over full length aa sequence and 99.8% identity in 1125 nucleotides. abstract</p> <p>-----</p>	

# INTERNATIONAL SEARCH REPORT

...ernational application No.  
PCT/US 01/30048

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 3  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 3

Present claims 17, 19, 22, and 25-26 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to nucleic acid probes and primers and to antibodies.

Present claim 3 relates to a compound defined by an intended reference to an ATCC Accession number. Said number, however, is missing in the claim and description which leads to lack of clarity within the meaning of Article 6 PCT. The lack of clarity is such as to render a meaningful search of claim 3 impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.